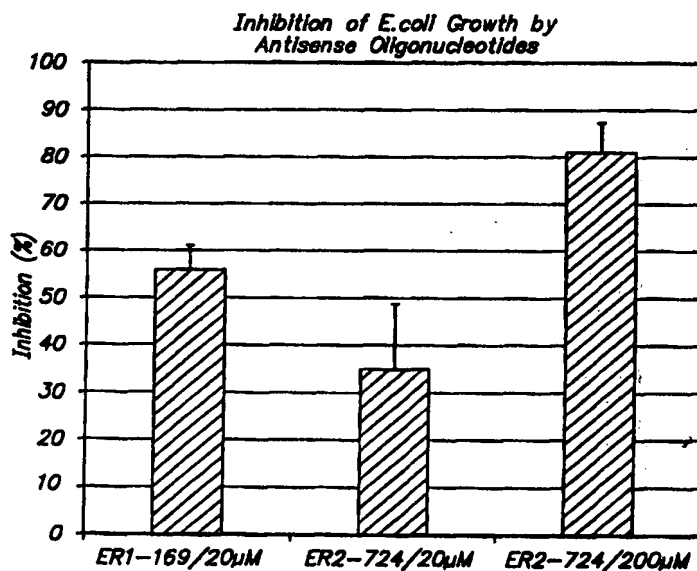




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(54) Title: ANTISENSE OLIGONUCLEOTIDE SEQUENCES AS INHIBITORS OF MICROORGANISMS



(57) Abstract

The invention relates to antisense oligonucleotides which modulate the expression of the ribonucleotide reductase or the secA genes in microorganisms. This invention is also related to methods of using such oligonucleotides in inhibiting the growth of microorganisms. These antisense oligonucleotides are particularly useful in treating pathological conditions in mammals which are mediated by the growth of microorganisms.

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ANTISENSE OLIGONUCLEOTIDE SEQUENCES AS INHIBITORS OF MICROORGANISMS

BACKGROUND OF THE INVENTION

5

Field of the Invention

This invention relates to antisense oligonucleotides which modulate the activity of the ribonucleotide reductase genes and the secA genes in microorganisms. This invention is also related to methods of using such compounds in inhibiting the growth of microorganisms.

These antisense oligonucleotides are particularly useful in treating pathological conditions in mammals which are mediated by the growth of microorganisms. Accordingly, this invention also relates to pharmaceutical compositions comprising a pharmaceutically acceptable excipient and an effective amount of a compound of this invention.

These antisense oligonucleotides may also be used as anti-microbial agents for agricultural applications such as crop protection.

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All of the above publications, patent applications and patents are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent application or patent was specifically and individually indicated to be incorporated by reference in its entirety.

State of the Art

Ribonucleotide reductase catalyzes the *de novo* production of deoxyribonucleotides. The enzyme reduces the four main ribonucleotides to the corresponding deoxyribonucleotides required for DNA synthesis and repair (Wright et al.⁴¹).

In mammalian and bacterial cells, *de novo* production of deoxyribonucleotides by ribonucleotide reductase is usually highly regulated on different levels in order to produce the correct amount of deoxyribonucleotides for DNA synthesis. In the DNA viruses, the metabolism of the host cell is directed towards production of viral DNA by virus encoded ribonucleotide reductases (Nordlund and Eklund¹).

Mammalian cells and many DNA viruses and prokaryotes, have a heterodimeric iron-containing ribonucleotide reductase enzyme of the $\alpha_2\beta_2$ type. For example, ribonucleotide reductase from *E. coli* is a multi-subunit $\alpha_2\beta_2$ enzyme where the two homo-dimeric proteins are denoted R1 and R2. The larger α_2 protein, R1, contains the binding sites for substrate and allosteric effectors and also the redox-active cysteine residues. Protein R1 has a molecular mass of 2 x 86,000 where each subunit contains 761 residues. The smaller β_2 protein, denoted R2, contains the dinuclear ferric center and a stable free tyrosyl radical necessary for the enzymatic activity. The R2 protein has a molecular mass of 2 x 43,500, where each subunit contains 375 amino acid residues (Nordlund and Eklund¹).

The nucleotide sequence of the *E. coli* K-12 DNA comprising the operon for the structural genes of the subunits of ribonucleotide reductase has been determined. The DNA sequence includes a total length of 8557 nucleotides. An open reading frame

between nucleotides 3506 and 5834 has been identified as the *nrdA* gene. An open reading frame between nucleotides 6012 and 7139 encoding a 375-amino acid polypeptide has been identified as the *nrdB* gene (Carlson et al.², and Nilsson et al.³). The sequences of the *nrdA* and *nrdB* genes for *E. coli* are shown in Figures 1 and 2.

5 In *E. coli*, the synthesis of ribonucleotide reductase is controlled at the level of transcription. The *nrdA* and *nrdB* genes direct the synthesis of a 3.2 kilobase polycistronic mRNA. Perturbations in DNA replication, either a shift up in growth conditions or an inhibition of DNA synthesis leads to increased synthesis of *nrd* mRNA (Carlson et al.²).

10 A separate anaerobic ribonucleotide reductase has also been identified from *E. coli*. The anaerobic *E. coli* reductase has a molecular mass of 145 kD and is a homodimer. The gene for the anaerobic reductase (*nrdD*) has been cloned and sequenced (P. Reichard⁴).

15 The ribonucleotide reductase R2 genomic or cDNA sequences are known for several other species such as bacteriophage T4, clam, mouse, *Saccharomyces cerevisiae*, vaccinia, herpes simplex virus types 1 and 2, varicella and Epstein-Barr virus (Nordlund et al.⁵). The sequence of the *nrdE* and *nrdF* which code for the ribonucleotide reductase genes of *S. typhimurium* are shown in Figure 3. The sequence of the ribonucleotide reductase gene of *Lactococcus lactis* is shown in Figure 4.

20 The *secA* gene of *E. coli* encodes for one component of a multi-component system for the secretion of proteins across the inner membrane of *E. coli* (der Blaauwen et al.⁶). The complete system consists of the SecB protein, a cytosolic chaperone, the SecA protein, the translocation ATPase and the heterotrimeric integral membrane SecY/SecE/SecG complex, which along with SecA serves as the preprotein
25 channel. SecA protein plays a central role in the secretion process by binding the preprotein, secB protein, anionic phospholipids and SecY/SecE/SecG protein. These interactions allow SecA to recognize soluble preprotein and recruit it to translocation sites in the inner membrane. Once such protein translocation complexes have assembled; further steps require an ATP-driven cycle of insertion and de-insertion of

secA protein with the inner membrane, where each cycle appears to be coupled to the translocation of a segment of the preprotein.

SecA is the only component of the secretion apparatus that has been shown to be regulated. SecA is the second gene in the geneX-secA operon and its translation varies over a tenfold range depending on the status of protein secretion in the cell. During protein-export proficient conditions secA auto-represses its translation by binding to a site that overlaps the secA ribosome-binding site of genes-secA RNA. SecA protein can also dissociate a preformed 30 S-tRNA^{MET}-genes-secA RNA ternary complex in vitro. However, during a protein export block secA translation increases substantially although the mechanism responsible for this regulatory response has not been elucidated (McNicholas et al.⁷). The sequence of the secA gene of *E. coli* is shown in Figure 5.

The secA gene sequence has been identified for a number of other species including *Mycobacterium bovis* (Figure 6), *Mycobacterium tuberculosis* (Figure 7), *Staphylococcus aureus* (Figure 8), *Staphylococcus carnosus* (Figure 9), *Bacillus subtilis*, *Bacillus firmus*, *Listeria monocytogenes*, *Mycobacterium smegmatis*, *Borrelia burgdorferi*, *P. sativum*, *S. griseus*, and *Synechoccus sp.*

Antibiotics are important pharmaceuticals for the treatment of infectious diseases in a variety of animals including man. The tremendous utility and efficacy of antibiotics results from the interruption of bacterial (prokaryotic) cell growth with minimal damage or side effects to the eukaryotic host harboring the pathogenic organisms. In general, antibiotics destroy bacteria by interfering with the DNA replication, DNA to RNA transcription, translation (that is RNA to protein) or cell wall synthesis.

Although bacterial antibiotic resistance has been recognized since the advent of antimicrobial agents, the consequence of the emergence of resistant microorganisms, such resistance was historically controlled by the continued availability of effective alternative drugs. Now, drug resistance has emerged as a serious medical problem in the community, leading to increasing morbidity and mortality. The problem is worsened by the growing number of pathogens resistant to multiple, structurally

unrelated drugs. The situation has become so desperate that antibiotics once removed from use because of toxic effects may be prescribed in an attempt to deal with the otherwise untreatable drug resistant bacteria.

Antisense oligonucleotides have been used to decrease the expression of specific
5 genes by inhibiting transcription or translation of the desired gene and thereby achieving a phenotypic effect based upon the expression of that gene (Wright and Anazado³⁸). For example, antisense RNA is important in plasmid DNA copy number control, in development of bacteriophage P22. Antisense RNA's have been used experimentally to specifically inhibit *in vitro* translation of mRNA coding specifically
10 from *Drosophila hsp23*, to inhibit Rous sarcoma virus replication and to inhibit 3T3 cell proliferation when directed toward the oncogene *c-fos*. Furthermore, it is not necessary to use the entire antisense mRNA since a short antisense oligonucleotide can inhibit gene expression. This is seen in the inhibition of chloramphenicol acetyltransferase gene expression and in the inhibition of specific antiviral activity to
15 vesicular stomatitis virus by inhibiting the N-protein initiation site. Antisense oligonucleotides directed to the macromolecular synthesis operon of bacteria, containing the *rpsU* gene, the *rpoD* gene and the *dnaG* gene have been used for the detection of bacteria. (U.S. Patent No. 5,294,533⁸). Furthermore, photoactivatable antisense DNA complementary to a segment of the β -lactamase gene has been used to
20 suppress ampicillin resistance in normally resistant *E. coli* (Gasparro et al.⁹). Antisense DNA analogs have also been used to inhibit the multiple antibiotic resistant (*mar*) operon in *Escherichia coli* (White et al.¹⁰).

Accordingly, there is a need to develop antisense oligonucleotides which will act to inhibit the growth of microorganisms.

25

SUMMARY OF THE INVENTION

This invention is directed to antisense oligonucleotides which modulate the expression of the ribonucleotide reductase and *secA* genes in microorganisms and pharmaceutical compositions comprising such antisense oligonucleotides. This

invention is also related to methods of using such antisense oligonucleotides for inhibiting the growth of microorganisms.

Accordingly, in one of its composition aspects, this invention is directed to an antisense oligonucleotide, which oligonucleotide is nuclease resistant and comprises
5 from about 3 to about 50 nucleotides, which nucleotides are complementary to the ribonucleotide reductase gene or the secA gene of a microorganism. The antisense oligonucleotide may have one or more phosphorothioate internucleotide linkages.

In another of its composition aspects, this invention is directed to an antisense oligonucleotide comprising from about 3 to about 50 nucleotides which is capable of
10 binding to the ribonucleotide reductase gene or the secA gene of a microorganism, wherein the oligonucleotide comprises all or part of a sequence selected from the group consisting of SEQ ID NO:22; SEQ ID NO:43; SEQ ID NO:62; SEQ ID NO:74; SEQ ID NO:75; SEQ ID NO:76; SEQ ID NO:143; SEQ ID NO:145; SEQ ID NO:152; SEQ ID NO:164; SEQ ID NO:176; SEQ ID NO:186; SEQ ID NO:188; SEQ ID
15 NO:189; SEQ ID NO:191; SEQ ID NO:192; SEQ ID NO:195; SEQ ID NO:197; SEQ ID NO:206; SEQ ID NO:212; SEQ ID NO:220; SEQ ID NO:229; SEQ ID NO:235; SEQ ID NO:254; SEQ ID NO:261; SEQ ID NO:262; SEQ ID NO:263; SEQ ID NO:264; and SEQ ID NO:265.

In still another of its composition aspects, this invention is directed to a
20 pharmaceutical composition comprising a pharmaceutically acceptable excipient and an effective amount of an antisense oligonucleotide, which oligonucleotide is nuclease resistant and comprises from about 3 to about 50 nucleotides, which nucleotides are complementary to the ribonucleotide reductase gene or the secA gene of a microorganism. The oligonucleotide may be modified, for example, the
25 oligonucleotide may have one or more phosphorothioate internucleotide linkages.

In one of its method aspects, this invention is directed to a method for inhibiting the expression of the ribonucleotide reductase gene in a microorganism having a ribonucleotide reductase gene comprising, administering to said microorganism or to a cell infected with said microorganism an effective amount of an antisense
30 oligonucleotide comprising from at least about 3 nucleotides which are complementary

to the ribonucleotide reductase gene of the microorganism under conditions such that the expression of the ribonucleotide reductase gene is inhibited.

In another of its method aspects, this invention is directed to a method for inhibiting the expression of the secA gene in a microorganism having a secA gene,
5 comprising administering to said microorganism an effective amount of an antisense oligonucleotide comprising from at least about 3 nucleotides which are complementary to the secA gene of the microorganism under conditions such that expression of the secA gene is inhibited.

In one of its method aspects, this invention is directed to a method for inhibiting
10 the growth of a microorganism encoding a ribonucleotide reductase gene or a secA gene, which method comprises administering to said microorganism or a cell infected with said microorganism an effective amount of an antisense oligonucleotide comprising from at least about 3 nucleotides which are complementary to either the ribonucleotide reductase gene or the secA gene of the microorganism under conditions
15 such that the growth of the microorganism is inhibited. Preferably, the antisense oligonucleotide is selected from the group consisting of SEQ ID NO:22; SEQ ID NO:43; SEQ ID NO:62; SEQ ID NO:74; SEQ ID NO:75; SEQ ID NO:76; SEQ ID NO:143; SEQ ID NO:145; SEQ ID NO:152; SEQ ID NO:164; SEQ ID NO:176; SEQ ID NO:186; SEQ ID NO:188; SEQ ID NO:189; SEQ ID NO:191; SEQ ID NO:192;
20 SEQ ID NO:195; SEQ ID NO:197; SEQ ID NO:206; SEQ ID NO:212; SEQ ID NO:220; SEQ ID NO:229; SEQ ID NO:235; SEQ ID NO:254; SEQ ID NO:261; SEQ ID NO:262; SEQ ID NO:263; SEQ ID NO:264; and SEQ ID NO:265.

In another of its method aspects, this invention is directed to a method for treating a mammalian pathologic condition mediated by a microorganism, which
25 method comprises identifying a mammal having a pathologic condition mediated by a microorganism having a ribonucleotide reductase gene or a secA gene and administering to said mammal an effective amount of an antisense oligonucleotide comprising from at least about 3 nucleotides which are complementary to either the ribonucleotide reductase gene or the secA gene of the microorganism under conditions
30 such that the growth of the microorganism is inhibited.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is the sequence of the *E. coli* nrdA gene encoding the ribonucleotide reductase R1 subunit [SEQ ID NO:1].

Figure 2 is the sequence of the *E. coli* nrdB gene encoding the ribonucleotide reductase R2 subunit [SEQ ID NO:2]. The nrdB gene is encoded by nucleotides 7668 to 8798 of SEQ ID NO:2.

Figure 3 is the sequence of the *S. typhimurium* nrdE and nrdF genes encoding the ribonucleotide reductase subunits [SEQ ID NO:3]. The nrdE gene is encoded by nucleotides 836 to 2980 and the nrdF gene is encoded by nucleotides 2991 to 3950 of SEQ ID NO:3.

Figure 4 is the sequence of the *Lactococcus lactis* nrdEF operon encoding ribonucleotide reductase [SEQ ID NO:4].

Figure 5 is the sequence of the *E. coli* secA gene [SEQ ID NO:5].

Figure 6 is the sequence of the *Mycobacterium bovis* secA gene [SEQ ID NO:6].

Figure 7 is the sequence of the *Mycobacterium tuberculosis* secA gene [SEQ ID NO:7].

Figure 8 is the sequence of the *Staphylococcus aureus* secA gene [SEQ ID NO:8].

Figure 9 is the sequence of the *Staphylococcus carnosus* secA gene [SEQ ID NO:9].

Figure 10 is the sequence of the bovine herpes virus ribonucleotide reductase small subunit gene [SEQ ID NO:10].

Figure 11 is the sequence of the Herpes simplex virus type 1 UL39 gene encoding ribonucleotide reductase 1 [SEQ ID NO:11].

Figure 12 is the sequence of the Herpes simplex type 2 ribonucleotide reductase gene [SEQ ID NO:12]. The ribonucleotide reductase gene is encoded by nucleotides 419 to 3853 of SEQ ID NO:12.

Figure 13 is the sequence of the equine herpes virus 4 ribonucleotide reductase large subunit and small subunit [SEQ ID NO:13]. The large subunit is encoded by

nucleotides 77 to 2446 and the small subunit by nucleotides 2485-3447 of SEQ ID NO:13.

Figure 14 is a photograph of a Western blot of a polyacrylamide gel of the cellular protein from *E. coli* cells carrying a plasmid containing the mouse
5 ribonucleotide reductase R2 gene after treatment with either 20 μ M or 200 μ M of oligonucleotide AS-II-626-20.

Figure 15 is a graph of the inhibition of *E. coli* growth after treatment of *E. coli* cells with ribonucleotide reductase antisense oligonucleotides.

Figure 16 is a graph of the number of colony forming units/ml of *E. coli* cells
10 after treatment with ribonucleotide reductase antisense oligonucleotides.

Figure 17 is a photograph of a Western blot of a polyacrylamide gel of cellular protein from *E. coli* cells after treatment with secA antisense oligonucleotides.

Figures 18a and 18b are graphs of the number of colony forming units/ml of *E. coli* cells after treatment with secA antisense oligonucleotides.

Figures 19a-g are graphs of growth curves of *E. coli* K12 after treatment with
15 antisense oligonucleotides. Figure 19a shows the growth after treatment with 16 μ M or 80 μ M of antisense ES799 [SEQ ID NO:195]. Figure 19b shows the growth after treatment with 20 μ M of antisense ES1739 [SEQ ID NO:229]. Figure 19c shows the growth after treatment with 80 μ M of antisense ES851 [SEQ ID NO:197]. Figure 19d
20 shows the growth after treatment with 80 μ M of antisense ES553 [SEQ ID NO:188]. Figure 19e shows the growth after treatment with 80 μ M of antisense ES646 [SEQ ID NO:191]. Figure 19f shows the growth after treatment with 80 μ M of antisense ES1845 [SEQ ID NO:235]. Figure 19g shows the growth after treatment with 80 μ M of antisense ES2537 [SEQ ID NO:254].

25

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides compounds that inhibit the growth of microbes by inhibiting the expression of a ribonucleotide reductase protein or the secA protein. Without being limited to any theory, the compounds inhibit the expression of the
30 ribonucleotide reductase or the secA protein by inhibiting the transcription of the gene

or the translation of the mRNA to protein. Such compounds include antisense oligonucleotides.

Definitions:

5 As used herein, the following terms have the following meanings:

The term "antisense oligonucleotide" as used herein means a nucleotide sequence that is complementary to the mRNA for the desired gene. Preferably, the antisense oligonucleotide is complementary to the mRNA for ribonucleotide reductase or secA.

10 The term "oligonucleotide" refers to an oligomer or polymer of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars, and inter-sugar (backbone) linkages. The term also includes modified or substituted oligomers comprising non-naturally occurring monomers or portions thereof, which function similarly. Such modified or substituted oligomers may be preferred over naturally
15 occurring forms because of the properties such as enhanced cellular uptake, or increased stability in the presence of nucleases. The term also includes chimeric oligonucleotides which contain two or more chemically distinct regions. For example, chimeric oligonucleotides may contain at least one region of modified nucleotides that confer beneficial properties (e.g. increased nuclease resistance, increased uptake into
20 cells) or two or more oligonucleotides of the invention may be joined to form a chimeric oligonucleotide.

The antisense oligonucleotides of the present invention may be ribonucleic or deoxyribonucleic acids and may contain naturally occurring or synthetic monomeric bases, including adenine, guanine, cytosine, thymine and uracil. The oligonucleotides
25 may also contain modified bases such as xanthine, hypoxanthine, 2-aminoadenine, 6-methyl, 2-propyl and other alkyl adenines, 5-halo uracil, 5-halo cytosine, 6-aza uracil, 6-aza cytosine and 6-aza thymine, pseudo uracil, 4-thiouracil, 8-halo adenine, 8-aminoadenine, 8-thiol adenine, 8-thiolalkyl adenines, 8-hydroxyl adenine and other 8-substituted adenines, 8-halo guanines, 8-amino guanine, 8-thiol guanine, 8-thioalkyl
30 guanines, 8-hydroxyl guanine and other 8-substituted guanines, other aza and deaza

uracils, thymidines, cytosines or guanines, 5-trifluoromethyl uracil and 5-trifluoro cytosine.

The antisense oligonucleotides of the invention may also comprise modified phosphorus oxygen heteroatoms in the phosphate backbone, short chain alkyl or
5 cycloalkyl intersugar linkages or short chain heteroatom or heterocyclic intersugar linkages. For example, the antisense oligonucleotides may contain methyl phosphonates, phosphorothioates, phosphorodithioates, phosphotriesters, and morpholino oligomers. In one embodiment of the invention, the antisense oligonucleotides comprise phosphorothioate bonds linking between the four to six 3'-
10 terminus nucleotides. In another embodiment, the phosphorothioate bonds link all the nucleotides. The antisense oligonucleotides may also have sugar mimetics.

The antisense oligonucleotides of the invention may also comprise nucleotide analogues wherein the structure of the nucleotide is fundamentally altered. An example of such an oligonucleotide analogue is a peptide nucleic acid (PNA) wherein the
15 deoxyribose (or ribose) phosphate backbone in DNA (or RNA) is replaced with a polyamide backbone which is similar to that found in peptides (Nielsen et al.¹¹; Good and Nielsen¹²; Buchardt, deceased, et al.¹³, U.S. Patent No. 5,766,855; Buchardt, deceased, et al.¹⁴, U.S. Patent No. 5,719,262). PNA analogues have been shown to be resistant to degradation by enzymes and to have extended lives *in vivo* and *in vitro*.
20 PNAs also bind more strongly to a complementary DNA sequence than to a naturally occurring nucleic acid molecule due to the lack of charge repulsion between the PNA strand and the DNA strand.

The oligonucleotides of the present invention may also include other nucleotides comprising polymer backbones, cyclic backbones, or acyclic backbones. For example,
25 the nucleotides may comprise morpholino backbone structures (U.S. Patent No. 5,034,506¹⁵).

The oligonucleotides of the present invention are "nuclease resistant" when they have either been modified such that they are not susceptible to degradation by DNA and RNA nucleases or alternatively they have been placed in a delivery vehicle which
30 in itself protects the oligonucleotide from DNA or RNA nucleases. Nuclease resistant

oligonucleotides include, for example, methyl phosphonates, phosphorothioates, phosphorodithioates, phosphotriesters, and morpholino oligomers. Suitable delivery vehicles for conferring nuclease resistance include, for example liposomes.

5 The oligonucleotides of the present invention may also contain groups, such as groups for improving the pharmacokinetic properties of an oligonucleotides, or groups for improving the pharmacodynamic properties of an oligonucleotide. Preferably, the oligonucleotides do not contain reporter groups or labels, such as fluorescent dyes or radioactive labels.

10 The antisense oligonucleotides may be complementary to the complete ribonucleotide reductase or secA gene including the introns. Preferably, the antisense oligonucleotides are complimentary to the mRNA region from the ribonucleotide reductase gene or the secA gene.

The antisense oligonucleotides may be selected from the sequence complementary to the ribonucleotide reductase or secA genes such that the sequence
15 exhibits the least likelihood of showing duplex formation, hair-pin formation, and homooligomer/sequence repeats but has a high to moderate potential to bind to the ribonucleotides reductase gene or the secA gene sequence and contains a GC clamp. These properties may be determined using the computer modeling program OLIGO Primer Analysis Software, Version 5.0 (distributed by National Biosciences, Inc.,
20 Plymouth, MN). This computer program allows the determination of a qualitative estimation of these five parameters.

Alternatively, the antisense oligonucleotides may also be selected on the basis that the sequence is highly conserved for either the ribonucleotide reductase or the secA genes between two or more microbial species. These properties may be determined
25 using the BLASTN program (Altschul, et al.¹⁶) of the University of Wisconsin Computer group (GCG) software (Devereux J. et al.¹⁷) with the National Center for Biotechnology Information (NCBI) databases.

The antisense oligonucleotides generally comprise from at least about 3 nucleotides or nucleotide analogs, preferably from about 3 to about 50 nucleotides or

nucleotide analogs, more preferably, from about 7 to about 35 nucleotides or nucleotide analogs, most preferably from about 15 to about 25 nucleotide or nucleotide analogs.

Preferably, the antisense oligonucleotides comprise from 3 to about 50 nucleotides or nucleotide analogs, more preferably from 20 to about 50 nucleotides or nucleotide analogs and further comprise all or part of the sequences set forth in Tables 1, 2, 3, and 4 (below). Preferably, the oligonucleotides complementary to the ribonucleotide reductase gene comprise SEQ ID NOS.: 14 to 157 as shown in Tables 1 and 2. Preferably, the antisense oligonucleotides complementary to the *secA* gene comprise the SEQ ID NOS.: 158 to 265 as shown in Tables 3 and 4.

Table 1

Antisense oligonucleotides that target the *Escherichia coli* K12 ribonucleotide reductase large subunit (R1)

SEQ ID No:	Name	Sequence 5'-3'	T _m (°C)	ΔG (kcal/mol)
14	ER1-16	CCGTCGCGCTTTGTCACCAG	61.1	-43.0
15	ER1-24	CTGTGCTACCGTCGCGCTTT	57.8	-42.0
16	ER1-33	TGATGCGCTCTGTGCTACCG	57.2	-40.2
17	ER1-44	TTTGTCGAGATTGAT GCGCT	53.3	-38.7
18	ER1-58	AGAACGCGATGGATTTTGTC	51.7	-38.4
19	ER1-71	TGCCGCCCAATCCAGAACGC	64.6	-46.0
20	ER1-79	AGTCCTTCTGCCGCCCAATC	57.7	-42.2
21	ER1-128	AAACTGAATGTGGGAGCGCA	55.5	-39.8
22	ER1-169	ATAATGGTTTCGTGGATGTC	55.5	-35.4
23	ER1-180	CGGCAGCCTTGATAATGGTT	54.2	-40.6
24	ER1-218	ATACTGATAATCCGGCGCAT	51.4	-39.4
25	ER1-252	TACGCAGGTGGAAGATCGCC	57.3	-41.4

SEQ ID No:	Name	Sequence 5'-3'	Tm (°C)	ΔG (kcal/mol)
26	ER1-294	GGTCGTACAGCGCAGGCGGC	64.4	-45.9
27	ER1-320	GCCCATCTCGACCATTTTCA	54.7	-39.7
28	ER1-330	TATCGTATTTGCCCATCTCG	50.4	-38.1
29	ER1-423	CGGCAGCATAAGAGAAGGTC	51.6	-38.5
5 30	ER1-439	CCTTCCAGCTGCTTAACGGC	56.4	-41.9
31	ER1-450	CCAGATATTTGCCTTCCAGC	51.5	-38.8
32	ER1-479	ATAGATTTGCGCGGTCACGC	56.4	-41.8
33	ER1-495	GGAAGTGGGCGCTCTCATAG	53.9	-39.7
34	ER1-504	GAATATAAAGGAACTGGGCG	48.5	-38.0
10 35	ER1-518	GCACGCGGCAACTAGAATAT	52.2	-39.4
36	ER1-529	TTCGAGAACAAGCACGCGGC	60.8	-43.3
37	ER1-543	TTTCACGCGGGTAGTTCGAG	55.2	-40.5
38	ER1-566	ACGCTTCACATATTGCAGGC	52.2	-38.7
39	ER1-584	GGAAACCGCGTCGTAAAAAC	53.9	-40.8
15 40	ER1-592	TTAAATGTGGAAACCGCGTC	52.7	-39.3
41	ER1-617	CATGATTGGCGTCGGCAGCG	64.0	-44.9
42	ER1-628	CGCACGCCGGACATGATTGG	63.8	-44.6
43	ER1-640	CGAGTCGGGGTACGCACGCC	64.2	-45.8
44	ER1-667	TCGATCAGTACGCAGGAGCT	52.4	-38.1
20 45	ER1-680	GCTGTCACCGCACTCGATCA	56.9	-39.1
46	ER1-689	GGAATCCAGGCTGTCACCGC	59.0	-41.9

SEQ ID No:	Name	Sequence 5'-3'	Tm (°C)	ΔG (kcal/mol)
47	ER1-704	GGAGGTGGCGTTGATGGAAT	56.0	-40.6
48	ER1-716	AACAATCGCGCTGGAGGTGG	59.5	-42.7
49	ER1-778	CTACCCAGCGCACGAATACG	55.7	-40.9
50	ER1-817	ATGCAGCCGGTATGGAACGC	59.4	-43.1
51	ER1-829	TTGTAGAACGGAATGCAGCC	52.8	-38.8
52	ER1-846	CCGCTGTCTGGAAATGTTTG	53.1	-38.6
53	ER1-855	AGGATTTACCGCTGTCTGG	54.0	-39.2
54	ER1-874	CGCACACCGCCCTGAGAGCA	63.9	-44.0
55	ER1-907	CACATCGGGTAGAACAGCGT	52.5	-38.1
56	ER1-925	CTTTCCACTTCCAGATGCCA	52.5	-38.1
57	ER1-964	TTGCCTTCCACACCACGGTT	57.5	-40.8
58	ER1-971	CACGCGGTTGCCTTCCACAC	60.8	-42.5
59	ER1-981	CCATATGACGCACGCGGTTG	59.4	-42.1
60	ER1-1034	TTCACCTTTCAGCAGACGGG	55.0	-39.6
61	ER1-1055	CGGGCTGAACAGGGTGATAT	53.8	-39.6
62	ER1-1059	CGGACGGGCTGAACAGGGTG	62.1	-43.7
63	ER1-1061	GTCGGACGGGCTGAACAGGG	61.2	-43.4
64	ER1-1106	AAACTCTTCCTGATCGGCGA	53.8	-39.7
65	ER1-1148	GCGGATGCTGTCGTCTTCT	54.3	-39.4
66	ER1-1155	GCTGCTTGCGGATGCTGTCG	61.3	-43.0
67	ER1-1166	GGCTTTCACACGCTGCTTGC	58.2	-41.4

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SEQ ID No:	Name	Sequence 5'-3'	Tm (°C)	ΔG (kcal/mol)
68	ER1-1173	GCTCAACGGCTTTCACACGC	58.0	-41.3
69	ER1-1212	GACCGGTAGACGCACGTTCC	56.7	-40.8
70	ER1-1255	GGGCTATGGGTATTGCAGTG	52.1	-38.7
71	ER1-1259	AAACGGGGCTATGGGTATTGC	53.3	-40.7
72	ER1-1265	CGGATCAAACGGGGCTATGGG	58.7	-43.4
73	ER1-1311	GGGCTATCTCCAGGCACAGG	55.9	-40.7
74	ER1-1315	GGCAGGGCTATCTCCAGGCA	58.7	-42.5
75	ER1-1320	TGGTCGGCAGGGCTATCTCC	58.6	-42.4
76	ER1-1326	GCGGTTTGGTCGGCAGGGCT	64.9	-47.0
77	ER1-1330	TTCAGCGGTTTGGTCGGCAG	60.5	-43.1
78	ER1-1336	ACGTCGTTACGCGGTTTGGT	56.8	-40.9
79	ER1-1356	TTTCACCGTTCTCGTCGTTG	53.5	-38.5
80	ER1-1364	CAGCGCGATTTACCGTTCT	57.5	-41.7
81	ER1-1370	CGTACACAGCGCGATTTAC	54.2	-38.9
82	ER1-1379	AGCAGACAGCGTACACAGCG	54.0	-38.2
83	ER1-1388	CAGGTTGAAAGCAGACAGCG	53.4	-38.4
84	ER1-1397	AATTGCGCCCAGGTTGAAAG	56.5	-41.9
85	ER1-1407	CCAGGTTATTAATTGCGCCC	53.8	-41.3
86	ER1-1428	TTGCCAGCTCTTCCAGTTCA	53.3	-38.2
87	ER1-1438	ACCGCCAGAATTGCCAGCTC	58.8	-42.5
88	ER1-1451	GTCAAGTGCACGAACCGCCA	59.1	-41.0

SEQ ID No:	Name	Sequence 5'-3'	Tm (°C)	ΔG (kcal/mol)
89	ER1-1463	ATCCAGCAGCGCGTCAAGTG	58.5	-41.2
90	ER1-1468	TGATAATCCAGCAGCGCGTC	56.1	-40.4
91	ER1-1535	GATCACACCAATACCCAGCG	52.6	-38.1
92	ER1-1561	TCGTTCGCCAGGTAGTAAGC	52.2	-39.0
5 93	ER1-1570	CGTTTACCGTCGTTTCGCCAG	57.9	-42.2
94	ER1-1584	TGCCGTCGGAGTAGCGTTTA	55.8	-41.0
95	ER1-1605	TATGCGTCAGGTTGTTGGCG	56.8	-40.5
96	ER1-1614	CGAAGGTTTTATGCGTCAGG	52.5	-39.3
97	ER1-1688	GTTAAACCACGGGCACGCGC	62.0	-45.0
10 98	ER1-1705	TTCGCGTAAGTGGTTTCGTT	52.6	-39.3
99	ER1-1731	TATAGGTATCGATCGGCAGG	49.5	-38.0
100	ER1-1777	CAGTCGTAATGCAGCGGCTC	55.8	-40.2
101	ER1-1789	CGCAGAGCTTCCCAGTCGTA	55.4	-40.0
102	ER1-1839	TCAGAGCAGAAAGCGTGGAG	53.0	-38.1
15 103	ER1-1849	TCGGACGGCATCAGAGCAGA	58.9	-40.9
104	ER1-1874	GGCGTTAGAGATCTGCGAAG	51.8	-38.7
105	ER1-1916	TTTGATGCTGACGTAACCGC	53.7	-39.0
106	ER1-1923	TCGACGCTTTGATGCTGACG	57.1	-40.2
107	ER1-1944	CCTGGCGCAAAATACCGTCT	56.5	-42.0
20 108	ER1-1957	TAGTCCGGCACCACCTGGCG	62.5	-44.2
109	ER1-1968	GCAGGTGCTCGTAGTCCGGC	59.3	-42.4

SEQ ID No:	Name	Sequence 5'-3'	Tm (°C)	ΔG (kcal/mol)
110	ER1-1974	CGTCGTGCAGGTGCTCGTAG	56.7	-39.9
111	ER1-1983	GCTCATAGGCGTCGTGCAGG	58.0	-41.4
112	ER1-1992	CCCACAGCAGCTCATAGGCG	58.0	-41.5
113	ER1-2000	CGGCATTTCCACAGCAGCT	59.7	-42.8
114	ER1-2010	CATCGTTACCCGGCATTTC	56.5	-41.9
115	ER1-2083	GGATCGTAGTTGGTGTGGC	51.8	-39.9
116	ER1-2112	TCGGCACTTTTCCTGACGGG	59.5	-42.8
117	ER1-2145	AGGCGGTGAGCAGGTCTTTC	55.7	-40.5
118	ER1-2154	CGAATTTGTAGGCGGTGAGC	54.8	-40.5
119	ER1-2166	GTGTTTTGACCCGAATTG	51.9	-38.6
120	ER1-2211	CGTCTTGTCGTCTTCAGCG	56.8	-40.0
121	ER1-2262	TCTTACATGCGCCGCTTTCG	58.6	-42.8

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Table 2

Antisense oligonucleotides that target the *Escherichia coli* K12 ribonucleotide reductase small subunit (R2)

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SEQ ID No:	Name	Sequence 5'-3'	Tm (°C)	ΔG (kcal/mol)
122	ER2-50	CGGCTGACCAAAGAACATCG	55.5	-40.0
123	ER2-60	CCACGTTGACCGGCTGACCA	61.2	-42.2
124	ER2-67	TAGCGAGCCACGTTGACCGG	60.6	-43.2
125	ER2-134	CGGACGCCAGAAGAAAGAGA	54.4	-39.8
126	ER2-144	CAACTTCTTCCGGACGCCAG	57.0	-41.3

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SEQ ID No:	Name	Sequence 5'-3'	Tm (°C)	ΔG (kcal/mol)
127	ER2-168	AATCTATACGGTCGCGGGAG	53.4	-40.5
128	ER2-198	TGTGTTTTTCGTGCTCCGGC	58.3	-41.6
129	ER2-273	GCAATAGCGCCACGTTCTGGG	62.1	-45.2
130	ER2-284	AGAAATAAGCGGCAATAGCG	51.8	-40.3
131	ER2-290	CGGAATAGAAATAAGCGGCA	52.4	-40.3
132	ER2-307	ACCCAGGTTTCCAGTTCCGG	57.4	-42.0
133	ER2-350	ATAGGAACGGGAATGAATCG	50.7	-38.8
134	ER2-441	TCCCTTCCGCACGTTTCTGG	59.5	-42.8
135	ER2-498	CGCCCAGCAGATGCCAGTAG	58.0	-41.5
136	ER2-505	GTACCTTCGCCCAGCAGATG	54.6	-39.7
137	ER2-544	CGCAGGCTAACGGTCACAGT	55.2	-39.7
138	ER2-557	TTTCTTCAGCTCGCGCAGGC	60.2	-43.4
139	ER2-640	GCAAATGCGAAGGAACAAGC	54.9	-40.4
140	ER2-655	ATCAATTCGCGTTCTGCAAA	53.4	-39.3
141	ER2-680	GCGAATAATTTTGGCGTTGC	54.9	-41.6
142	ER2-692	GCGGGCAATCAGGCGAATAA	59.5	-44.0
143	ER2-704	CAGGGCTTCGTCTCGGGCAA	66.8	-47.8
144	ER2-714	CGGTCAGGTGCAGGGCTTCG	62.3	-44.0
145	ER2-724	TGCTGGGTGCCGGTCAGGTG	63.6	-43.5
146	ER2-728	CATATGCTGGGTGCCGGTCA	58.8	-41.4
147	ER2-778	GCAATTTCCGCCATCTCAGG	56.8	-41.5

SEQ ID No:	Name	Sequence 5'-3'	Tm (°C)	ΔG (kcal/mol)
148	ER2-796	TCCTGCTTACACTCTTCGGC	52.1	-38.3
149	ER2-848	ATCCGCCCAGTCTTTCTCCT	54.2	-40.4
150	ER2-857	GAACAGATAATCCGCCCAGT	50.7	-38.1
151	ER2-976	GGGTTGGAGCGCGTCTGGAA	61.8	-44.0
152	ER2-983	CGGGATCGGGTTGGAGCGCG	68.1	-49.1
153	ER2-985	CACGGGATCGGGTTGGAGCG	64.0	-45.6
154	ER2-1045	CTGACTTCCACTTCCTGCGG	54.6	-39.9
155	ER2-1063	TGCCCCGACCAGATAAGAACT	51.3	-38.2
156	ER2-1076	TTCCGAGTCAATCTGCCCGA	57.8	-41.2
157	ER2-1092	AATCGTCGGTGTCCACTTCC	53.6	-38.8

Table 3
Antisense Sequences that Target *Escherichia coli* SecA

SEQ ID No:	Name	Sequence 5 - 3'	Tm (°C)	ΔG kDa/mol
158	ES56	GACCACTTTGCGCATCCGGC	62.1	-44.2
159	ES62	GATGTTGACCACTTTGCGCA	54.3	-38.3
160	ES85	ATCTCCGGTTCCATGGCATT	55.5	-40.8
161	ES92	TTTTTCCATCTCCGGTTCCA	54.3	-40.1
162	ES116	CCCTTTCAGTTCTTCGTCGG	53.8	-39.8
163	ES124	GCGGTTTTCCCTTTCAGTTC	52.9	-39.9
164	ES129	ACTCTGCGGTTTTCCCTTTC	52.5	-39.6
165	ES153	CGCCTTTTTCCAGACGTGCA	58.4	-41.9
166	ES158	CACTTCGCCTTTTTCCAGAC	51.5	-38.4
167	ES165	TTTCCAGCACTTCGCCTTTT	54.1	-40.5

	SEQ ID No:	Name	Sequence 5 ~ 3'	Tm (°C)	ΔG kDa/mol
5	168	ES170	CAGATTTTCCAGCACTTCGC	52.5	-38.6
	169	ES206	ACTTGCCTCACGTACCACGG	54.9	-39.5
	170	ES215	GACGCGCTTACTTGCCTCAC	55.0	-40.1
	171	ES230	GTGACGCATACCAAAGACGC	53.1	-38.5
	172	ES264	TAAGAACCATAACCGCCGAGT	51.5	-39.1
10	173	ES286	ATTTTCGGCGATGCAGCGTTC	59.7	-43.4
	174	ES303	TTCCTTCACCGGTACGCATT	54.5	-40.3
	175	ES307	GTTTTTCCTTCACCGGTACG	51.4	-38.9
	176	ES320	CGTTGCGGTCAGGGTTTTTC	56.8	-41.6
	177	ES336	TCAGGTAAGCAGGCAGCGTT	55.0	-40.2
15	178	ES351	TACCGGTTAGTGCGTTCAGG	52.8	-39.2
	179	ES392	TTGCGCCAGGTAGTCGTTGA	56.5	-40.4
	180	ES398	GTCACGTTGCGCCAGGTAGT	55.0	-39.5
	181	ES418	AGCGGACGGTTGTTTTCGGC	60.8	-44.5
	182	ES429	GGAATTCAAACAGCGGACGG	56.7	-41.5
20	183	ES436	AGGCCAAGGAATTCAAACAG	51.0	-38.4
	184	ES448	ATACCGACAGTCAGGCCAAG	51.6	-38.0
	185	ES485	TTCGCGCTTTGCCGGTGCTG	65.8	-46.9
	186	ES531	AGCCGTATTCGTTGTTCGTA	50.1	-37.9
	187	ES544	CGCAGGTAGTCAAAGCCGTA	53.1	-39.5
25	188	ES553	ATGTTGTCGCGCAGGTAGTC	52.6	-38.1
	189	ES556	GCCATGTTGTCGCGCAGGTA	59.2	-41.7
	190	ES617	GTCCACTTCGTCCACCAGCG	57.7	-40.4
	191	ES646	GGTGTACGCGCTTCATCGAT	55.0	-40.0
	192	ES647	CGGTGTACGCGCTTCATCGA	59.3	-42.1
	193	ES695	GCGTTTATACATTTCCGAGC	49.5	-38.4
	194	ES724	CGGATCAGGTGCGGAATAAT	53.9	-40.4

SEQ ID No:	Name	Sequence 5' - 3'	Tm (°C)	ΔG kDa/mol
195	ES799	TTCACCTGGCGAGATTTTTC	51.8	-38.6
196	ES824	CAGCACCAGACCACGTTTCGG	58.6	-40.7
197	ES851	GCCCTCTTTCACCAGCAGTT	53.3	-39.1
198	ES866	CCCTTCATCCATGATGCCCT	55.9	-40.6
199	ES889	TTGGCCGGAGAGTACAGAGA	52.2	-38.1
200	ES898	AGCATGATGTTGGCCGGAGA	57.6	-40.9
201	ES922	AGCGCCGCCGTTACGTGGTG	64.6	-46.5
202	ES950	GTCACGGGTAAACAGCGCAT	54.9	-40.0
203	ES1068	CACCTTCTTTCGCTTCCACA	52.8	-38.4
204	ES1097	CAGCGTTTGGTTTTTCGTTCT	52.1	-38.9
205	ES1109	GGTGATCGAAGCCAGCGTTT	56.5	-41.2
206	ES1128	GACGGAAGTAGTTCTGGAAG	45.5	-35.0
207	ES1147	CCCGCCAGTTTTTTCATACAG	52.3	-39.2
208	ES1152	TCATCCCCGCCAGTTTTTCA	57.5	-41.6
209	ES1218	GAACAACGACGGTATCCAGC	52.0	-38.2
210	ES1328	GCCTTTCGCAGTACGTTCTT	51.4	-38.9
211	ES1350	TAGTACCCACCAGCACCGGC	57.1	-41.4
212	ES1398	CGGCTTTGGTCAGTTCGTTT	54.3	-40.1
213	ES1410	TGTGCTTAATACCGGCTTTG	50.8	-38.6
214	ES1439	GTTGGCGTGGAATTTGGCGT	59.3	-43.0
215	ES1462	GCCTGAGCAACAATCGCCGC	62.4	-44.5
216	ES1515	CTGTACCACGACCCGCCATA	55.6	-40.3
217	ES1518	TATCTGTACCACGACCCGCC	54.7	-40.0
218	ES1545	CTGCCTGCCAGCTACCACCG	60.2	-42.9
219	ES1563	TTTCCAGCGCGGCAACTTCT	59.4	-43.4
220	ES1581	TTTGCTCTGCGGTCGGATTT	57.0	-41.8
221	ES1589	TTTTTCAATTTGCTCTGCGG	53.2	-39.8

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SEQ ID No:	Name	Sequence 5 - 3'	Tm (°C)	ΔG kDa/mol
222	ES1624	ACCGCATCGTGACGTACCTG	55.7	-39.6
223	ES1629	CCAGTACCGCATCGTGACGT	55.7	-39.6
224	ES1633	GCTTCCAGTACCGCATCGTG	55.5	-40.0
225	ES1655	ACCGATGATATGCAGGCCAC	54.6	-39.6
226	ES1712	ACGACCAGAACGACCGCGCA	63.3	-44.1
227	ES1718	CCCCTGACGACCAGAACGAC	56.6	-40.1
228	ES1722	CATCCCCCTGACGACCAGAA	56.9	-40.4
229	ES1739	GAAACGGGAAGAACCAGCAT	53.1	-39.5
230	ES1748	CGACAGGTAGAAACGGGAAG	51.4	-38.6
231	ES1781	GGAAGCAAAAATACGCATCA	50.6	-38.2
232	ES1785	GGTCGGAAGCAAAAATACGC	53.9	-40.9
233	ES1794	CGGATACTCGGTCGGAAGCA	57.3	-41.7
234	ES1814	ACCCAGTTTACGCATCATGC	52.5	-38.5
235	ES1845	ACGGGTGTTCAATGGCTTCG	57.1	-41.2
236	ES1861	ATCGCTTTAGTCACCCACGG	54.1	-40.0
237	ES1888	CTTTCAACTTTACGCTGGGC	51.9	-39.3
238	ES1892	ACGGCTTTCAACTTTACGCT	51.1	-39.2
239	ES2007	TGGTTTCGCTCACATCGCTG	57.0	-40.0
240	ES2054	GTAGGCATCAATGGTCGCTT	51.7	-38.5
241	ES2084	CCACATTTCTTCCAGCGACT	51.7	-38.0
242	ES2087	ATCCCACATTTCTTCCAGCG	53.9	-39.7
243	ES2191	TCACGCAGCGTCTCTTCATG	54.7	-38.2
244	ES2275	CCTTTCTCGAAGTGACGCAT	51.9	-38.2
245	ES2306	CCACAGGGAGTCAAGCGTTT	54.1	-39.3
246	ES2325	TCGCTGCCAGGTGCTCTTTC	57.7	-41.1
247	ES2330	GTCCATCGCTGCCAGGTGCT	59.7	-41.9
248	ES2339	ACGCAGATAGTCCATCGCTG	52.7	-38.4

SEQ ID No:	Name	Sequence 5 - 3'	Tm (°C)	ΔG kDa/mol
249	ES2381	CTTCGGATCTTTCTGTGCGT	51.9	-38.2
250	ES2395	CGTTTGTATTCTGCTTCGG	52.5	-39.4
251	ES2422	ATCGCTGCAAACATGGAGAA	53.1	-38.5
252	ES2520	CCATACGACGCTGTTGTTC	52.9	-38.5
253	ES2525	GGCTTCCATACGACGCTGTT	54.2	-40.0
254	ES2537	CGCTAAACGCTCGGCTTCCA	59.9	-44.1
255	ES2555	GCTAAGCTGCTGCATTTGCG	56.2	-41.3
256	ES2619	CTACTTTGCGCTCTCCGGTT	53.8	-40.4
257	ES2626	TTACGTCCTACTTTGCGCTC	50.0	-38.0
258	ES2646	AACCGCACGGGCAAGGATCG	63.6	-45.9
259	ES2651	ACCAGAACCGCACGGGCAAG	61.7	-44.0
260	ES2656	TTTTTACCAGAACCGCACGG	55.1	-41.0

Table 4
Antisense Sequences that Target *E. coli* *SecA* based on Conserved Sequences

SEQ ID No:	Name	Sequence 5 - 3'	Tm (°C)	ΔG kDa/mol
261	ES386	CAGGTAGTCGTTGACGGTAA	47.7	-35.7
262	ES388	CAGGTAGTCGTTGACGGT	45.0	-32.9
263	ES1126	CGGAAGTAGTTCTGGAAGGT	47.6	-36.5
264	ES1702	CGACCGCGCAACTGGTTATC	57.8	-41.9
265	ES2644	CCGCACGGGCAAGGATCGTT	63.6	-45.9

In Tables 1, 2, 3, and 4, the "Tm" is the melting temperature of an oligonucleotide duplex calculated according to the nearest-neighbor thermodynamic values. At this temperature 50% of nucleic acid molecules are in duplex and 50% are denatured. The "ΔG" is the free energy of the oligonucleotide, which is a measurement of an oligonucleotide duplex stability.

The following sequences have been determined to be conserved among species:

ES386 [SEQ ID NO:261] is conserved among *Escherichia coli* and
Mycobacterium tuberculosis;

ES388 [SEQ ID NO:262] is conserved among *Escherichia coli*; *Mycobacterium*
5 *tuberculosis*; and *Mycobacterium bovis*;

ES553 [SEQ ID NO:188] is conserved among *Escherichia coli*, *Mycobacterium*
tuberculosis, *Mycobacterium bovis*, *Streptomyces coelicolor*; and *Streptomyces lividans*;

ES556 [SEQ ID NO:189] is conserved among *Escherichia coli*, *Mycobacterium*
tuberculosis, *Mycobacterium bovis*, *Streptomyces coelicolor*; and *Streptomyces lividans*;
10 and *Synechococcus sp.*; and

ES646 [SEQ ID NO:191] is conserved among *Escherichia coli* and
Staphylococcus carnosus;

ES1126 [SEQ ID NO:263] is conserved among *Escherichia coli* and
Rhodobacter capsulatus SecA genes.

ES2644 [SEQ ID NO:265] is conserved among *Escherichia coli* SecA gene,
15 MutA (A:T to C:G transversion), and tyrosine-specific transport protein (tyrP) gene.

The term "alkyl" refers to monovalent alkyl groups preferably having from 1 to
20 carbon atoms and more preferably 1 to 6 carbon atoms. This term is exemplified
by groups such as methyl, ethyl, *n*-propyl, *iso*-propyl, *n*-butyl, *iso*-butyl, *n*-hexyl, and
20 the like.

The term "aryl" refers to an unsaturated aromatic carbocyclic group of from 6
to 14 carbon atoms having a single ring (e.g., phenyl) or multiple condensed (fused)
rings (e.g., naphthyl or anthryl). Preferred aryls include phenyl, naphthyl and the like.

The term "cycloalkyl" refers to cyclic alkyl groups of from 3 to 20 carbon
25 atoms having a single cyclic ring or multiple condensed rings. Such cycloalkyl groups
include, by way of example, single ring structures such as cyclopropyl, cyclobutyl,
cyclopentyl, cyclooctyl, and the like, or multiple ring structures such as adamantanyl,
and the like.

The term "halo" or "halogen" refers to fluoro, chloro, bromo and iodo and
30 preferably is either fluoro or chloro.

The term "thiol" refers to the group -SH.

As to any of the above groups which contain one or more substituents, it is understood, of course, that such groups do not contain any substitution or substitution patterns which are sterically impractical and/or synthetically non-feasible. In addition,
5 the compounds of this invention include all stereochemical isomers arising from the substitution of these compounds.

The term "pharmaceutically acceptable salt" refers to salts which retain the biological effectiveness and properties of the antisense oligonucleotides of this invention and which are not biologically or otherwise undesirable. In many cases, the
10 antisense oligonucleotides of this invention are capable of forming acid and/or base salts by virtue of the presence of amino and/or carboxyl groups or groups similar thereto.

Pharmaceutically acceptable base addition salts can be prepared from inorganic and organic bases. Salts derived from inorganic bases, include by way of example
15 only, sodium, potassium, lithium, ammonium, calcium and magnesium salts. Salts derived from organic bases include, but are not limited to, salts of primary, secondary and tertiary amines, such as alkyl amines, dialkyl amines, trialkyl amines, substituted alkyl amines, di(substituted alkyl) amines, tri(substituted alkyl) amines, alkenyl amines, dialkenyl amines, trialkenyl amines, substituted alkenyl amines, di(substituted
20 alkenyl) amines, tri(substituted alkenyl) amines, cycloalkyl amines, di(cycloalkyl) amines, tri(cycloalkyl) amines, substituted cycloalkyl amines, disubstituted cycloalkyl amine, trisubstituted cycloalkyl amines, cycloalkenyl amines, di(cycloalkenyl) amines, tri(cycloalkenyl) amines, substituted cycloalkenyl amines, disubstituted cycloalkenyl amine, trisubstituted cycloalkenyl amines, aryl amines, diaryl amines, triaryl amines,
25 heteroaryl amines, diheteroaryl amines, triheteroaryl amines, heterocyclic amines, diheterocyclic amines, triheterocyclic amines, mixed di- and tri-amines where at least two of the substituents on the amine are different and are selected from the group consisting of alkyl, substituted alkyl, alkenyl, substituted alkenyl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, aryl, heteroaryl,

heterocyclic, and the like. Also included are amines where the two or three substituents, together with the amino nitrogen, form a heterocyclic or heteroaryl group.

Examples of suitable amines include, by way of example only, isopropylamine, trimethyl amine, diethyl amine, tri(*iso*-propyl) amine, tri(*n*-propyl) amine, 5 ethanolamine, 2-dimethylaminoethanol, tromethamine, lysine, arginine, histidine, caffeine, procaine, hydrabamine, choline, betaine, ethylenediamine, glucosamine, N-alkylglucamines, theobromine, purines, piperazine, piperidine, morpholine, N-ethylpiperidine, and the like. It should also be understood that other carboxylic acid derivatives would be useful in the practice of this invention, for example, carboxylic 10 acid amides, including carboxamides, lower alkyl carboxamides, dialkyl carboxamides, and the like.

Pharmaceutically acceptable acid addition salts may be prepared from inorganic and organic acids. Salts derived from inorganic acids include hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, and the like. Salts 15 derived from organic acids include acetic acid, propionic acid, glycolic acid, pyruvic acid, oxalic acid, malic acid, malonic acid, succinic acid, maleic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, *p*-toluene-sulfonic acid, salicylic acid, and the like.

The term "ribonucleotide reductase gene" or the "ribonucleoside diphosphate 20 reductase gene" refers to any gene which encodes a protein that either reduces the four main ribonucleotides to the corresponding deoxyribonucleotides involved in DNA synthesis or encodes a subunit of a multimeric enzyme which reduces the four main ribonucleotides to the corresponding deoxyribonucleotides. Without being limiting, examples of ribonucleotide reductase genes from bacteria include the *E. coli* *nrdA*, 25 *nrdB* and *nrdD* genes; the *S. typhimurium* *nrdE* and *nrdF* genes; and the *Lactococcus lactis* *nrdEF* gene. Examples of the ribonucleotide reductase genes from viruses include the herpes simplex type 1 and 2 ribonucleotide reductases and the bovine and equine herpes simplex ribonucleotide reductases.

The term "secA" refers to an oligonucleotide sequence which encodes a protein 30 having similar properties as those expressed by the *E. coli* *secA* gene. Without being

limiting, examples of secA genes from bacteria include the *Mycobacterium bovis* secA gene; the *Mycobacterium tuberculosis* secA gene, the *Staphylococcus aureus* secA gene and the *Staphylococcus carnosus* secA gene.

5 The term "microorganism" means a bacteria, fungi or virus having either a ribonucleotide reductase or secA gene. Specifically excluded from this definition is the malarial parasite, plasmodium.

The term "bacteria" refers to any bacteria encoding either a ribonucleotide reductase gene or a secA gene, including *Escherichia coli*, *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium smegmatis*, *Salmonella typhimurium*,
10 *Thermoplasma acidophilum*, *Pyrococcus furiosus*, *Bacillus subtilis*, *Bacillus firmus*, *Lactococcus lactis*, *Staphylococcus aureus*, *Staphylococcus carnosus*, *Listeria monocytogenes*, *Borrelia burgdorferi*, *P. sativum*, *S. griseus*, and *Synechococcus sp.*

The term "virus" refers to any virus having a ribonucleotide reductase gene. Preferably the virus will be a DNA virus. Examples of suitable viruses include various
15 herpes viruses (such as herpes simplex types 1 and 2, varicella-herpes zoster, cytomegalovirus and Epstein-Barr virus) and the various hepatitis viruses.

The term "complementary to" means that the antisense oligonucleotide sequence is capable of binding to the target sequence, ie the ribonucleotide reductase gene or the secA gene. Preferably the antisense oligonucleotide sequence has at least about 75%
20 identity with the target sequence, preferably at least about 90% identity and most preferably at least about 95% identity with the target sequence allowing for gaps or mismatches of several bases. Identity can be determined, for example, by using the BLASTN program of the University of Wisconsin Computer Group (GCG) software.

The term "inhibiting growth" means a reduction in the growth of the bacteria or
25 viruses of at least 25%, more preferably of at least 50% and most preferably of at least 75%. The reduction in growth can be determined for bacteria by measuring the optical density of a liquid bacteria culture with a spectrophotometer or by counting the number of colony forming units/ml (CFU/ml) upon plating on culture plates. The reduction in growth can be determined for viruses by measuring the number of plaque
30 forming units/ml upon plating on susceptible cells.

Preparation of the Antisense Oligonucleotides

The antisense oligonucleotides of the present invention may be prepared by conventional and well-known techniques. For example, the oligonucleotides may be prepared using solid-phase synthesis and in particular using commercially available
5 equipment such as the equipment available from Applied Biosystems Canada Inc., Mississauga, Canada. The oligonucleotides may also be prepared by enzymatic digestion of the naturally occurring ribonucleotide reductase or secA gene by methods known in the art.

10 Isolation and Purification of the Antisense Oligonucleotides

Isolation and purification of the antisense oligonucleotides described herein can be effected, if desired, by any suitable separation or purification such as, for example, filtration, extraction, crystallization, column chromatography, thin-layer
15 chromatography, thick-layer chromatography, preparative low or high-pressure liquid chromatography or a combination of these procedures. However, other equivalent separation or isolation procedures could, of course, also be used.

The invention contemplates a method of evaluating if an antisense oligonucleotide inhibits the growth of a microbe having a ribonucleotide reductase or secA gene. The method comprises selecting the microbe/microorganism having a
20 ribonucleotide reductase or secA gene, administering the antisense oligonucleotide; and comparing the growth of the treated microbe with the growth of an untreated microorganism.

In order for the antisense oligonucleotide to effectively interrupt the expression of the ribonucleotide reductase or secA gene, the antisense oligonucleotide enters the
25 microorganism's cell, in the case of fungal or bacterial cells or enter the mammalian cell having the virus target.

Although oligonucleotides are taken up by bacterial cells, some modification of the oligonucleotides may help facilitate or regulate said uptake. thus, a carrier molecule, for example an amino acid, can be linked to the oligonucleotide. for
30 example, bacteria have multiple transport systems for the recognition and uptake of

molecules of leucine. The addition of this amino acid to the oligonucleotide may facilitate the uptake of the oligonucleotide in the bacteria and not substantially interfere with the activity of the antisense oligonucleotide in the bacterial cell.

Other methods are contemplated for facilitating the uptake of the antisense oligonucleotide into bacteria. For example, the addition of other amino acids or peptides or primary amines to the 3' or 5' termini of the antisense oligonucleotide may enable utilization of specific transport systems. Addition of lactose to the oligonucleotide by a covalent linkage may also be used to enable transport of the antisense oligonucleotide by lactose permease. Other sugar transport systems are also known to be functional in bacteria and can be utilized in this invention.

With regard to inhibiting the expression of ribonucleotide reductase in DNA viruses, the antisense oligonucleotide is preferably introduced into the cell infected with the DNA virus. The antisense oligonucleotides may be delivered using vectors or liposomes.

An expression vector comprising the antisense oligonucleotide sequence may be constructed having regard to the sequence of the oligonucleotide and using procedures known in the art. The vectors may be selected from plasmids or benign viral vectors depending on the eukaryotic cell and the DNA virus. Phagemids are a specific example of beneficial vectors because they can be used either as plasmids or a bacteriophage vectors. Examples of other vectors include viruses such as bacteriophages, baculoviruses and retroviruses, DNA viruses, liposomes and other recombination vectors.

Vectors can be constructed by those skilled in the art to contain all the expression elements required to achieve the desired transcription of the antisense oligonucleotide sequences. Therefore, the invention provides vectors comprising a transcription control sequence operatively linked to a sequence which encodes an antisense oligonucleotide. Suitable transcription and translation elements may be derived from a variety of sources, including bacterial, fungal, viral, mammalian or insect genes. Selection of appropriate elements is dependent on the host cell chosen.

Reporter genes may be included in the vector. Suitable reporter genes include β -galactosidase (e.g. lacZ), chloramphenicol, acetyl-transferase, firefly luciferase, or an immunoglobulin or portion thereof. Transcription of the antisense oligonucleotide may be monitored by monitoring for the expression of the reporter gene.

5 The vectors can be introduced into cells or tissues by any one of a variety of known methods within the art. Such methods can be found generally described in Sambrook et al.¹⁸; Ausubel et al.¹⁹; Chang et al.²⁰; Vega et al.²¹; and Vectors: A Survey of Molecular Cloning Vectors and Their Uses²² and include, for example, stable or transient transfection, lipofection, electroporation and infection with
10 recombinant viral vectors.

Introduction of nucleic acids by infection offers several advantages. Higher efficiency and specificity for tissue type can be obtained. Viruses typically infect and propagate in specific cell types. Thus, the virus' specificity may be used to target the vector to specific cell types *in vivo* or within a tissue or mixed culture of cells. Viral
15 vectors can also be modified with specific receptors or ligands to alter target specificity through receptor mediated events.

Pharmaceutical Formulations

When employed as pharmaceuticals, the antisense oligonucleotides are usually
20 administered in the form of pharmaceutical compositions. These compounds can be administered by a variety of routes including oral, rectal, transdermal, subcutaneous, intravenous, intramuscular, and intranasal. These compounds are effective as both injectable and oral compositions. Such compositions are prepared in a manner well known in the pharmaceutical art and comprise at least one active compound.

25 This invention also includes pharmaceutical compositions which contain, as the active ingredient, one or more of the antisense oligonucleotides associated with pharmaceutically acceptable carriers. In making the compositions of this invention, the active ingredient is usually mixed with an excipient, diluted by an excipient or enclosed within such a carrier which can be in the form of a capsule, sachet, paper or other
30 container. When the excipient serves as a diluent, it can be a solid, semi-solid, or

liquid material, which acts as a vehicle, carrier or medium for the active ingredient. Thus, the compositions can be in the form of tablets, pills, powders, lozenges, sachets, cachets, elixirs, suspensions, emulsions, solutions, syrups, aerosols (as a solid or in a liquid medium), ointments containing, for example, up to 10% by weight of the active compound, soft and hard gelatin capsules, suppositories, sterile injectable solutions, and sterile packaged powders.

In preparing a formulation, it may be necessary to mill the active compound to provide the appropriate particle size prior to combining with the other ingredients. If the active compound is substantially insoluble, it ordinarily is milled to a particle size of less than 200 mesh. If the active compound is substantially water soluble, the particle size is normally adjusted by milling to provide a substantially uniform distribution in the formulation, e.g. about 40 mesh.

Some examples of suitable excipients include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, tragacanth, gelatin, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, sterile water, syrup, and methyl cellulose. The formulations can additionally include: lubricating agents such as talc, magnesium stearate, and mineral oil; wetting agents; emulsifying and suspending agents; preserving agents such as methyl- and propylhydroxy-benzoates; sweetening agents; and flavoring agents. The compositions of the invention can be formulated so as to provide quick, sustained or delayed release of the active ingredient after administration to the patient by employing procedures known in the art.

The compositions are preferably formulated in a unit dosage form, each dosage containing from about 5 to about 100 mg, more usually about 10 to about 30 mg, of the active ingredient. The term "unit dosage forms" refers to physically discrete units suitable as unitary dosages for human subjects and other mammals, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect, in association with a suitable pharmaceutical excipient. Preferably, the antisense oligonucleotide is employed at no more than about 20 weight percent of

the pharmaceutical composition, more preferably no more than about 15 weight percent, with the balance being pharmaceutically inert carrier(s).

The antisense oligonucleotide is effective over a wide dosage range and is generally administered in a pharmaceutically effective amount. It, will be understood, however, that the amount of the antisense oligonucleotide actually administered will be determined by a physician, in the light of the relevant circumstances, including the condition to be treated, the chosen route of administration, the actual compound administered, the age, weight, and response of the individual patient, the severity of the patient's symptoms, and the like.

For preparing solid compositions such as tablets, the principal active ingredient/antisense oligonucleotide is mixed with a pharmaceutical excipient to form a solid preformulation composition containing a homogeneous mixture of a compound of the present invention. When referring to these preformulation compositions as homogeneous, it is meant that the active ingredient is dispersed evenly throughout the composition so that the composition may be readily subdivided into equally effective unit dosage forms such as tablets, pills and capsules. This solid preformulation is then subdivided into unit dosage forms of the type described above containing from, for example, 0.1 to about 500 mg of the active ingredient of the present invention.

The tablets or pills of the present invention may be coated or otherwise compounded to provide a dosage form affording the advantage of prolonged action. For example, the tablet or pill can comprise an inner dosage and an outer dosage component, the latter being in the form of an envelope over the former. The two components can be separated by an enteric layer which serves to resist disintegration in the stomach and permit the inner component to pass intact into the duodenum or to be delayed in release. A variety of materials can be used for such enteric layers or coatings, such materials including a number of polymeric acids and mixtures of polymeric acids with such materials as shellac, cetyl alcohol, and cellulose acetate.

The liquid forms in which the novel compositions of the present invention may be incorporated for administration orally or by injection include aqueous solutions, suitably flavored syrups, aqueous or oil suspensions, and flavored emulsions with

edible oils such as corn oil, cottonseed oil, sesame oil, coconut oil, or peanut oil, as well as elixirs and similar pharmaceutical vehicles.

Compositions for inhalation or insufflation include solutions and suspensions in pharmaceutically acceptable, aqueous or organic solvents, or mixtures thereof, and
5 powders. The liquid or solid compositions may contain suitable pharmaceutically acceptable excipients as described *supra*. Preferably the compositions are administered by the oral or nasal respiratory route for local or systemic effect. Compositions in preferably pharmaceutically acceptable solvents may be nebulized by use of inert gases. Nebulized solutions may be inhaled directly from the nebulizing device or the
10 nebulizing device may be attached to a face mask tent, or intermittent positive pressure breathing machine. Solution, suspension, or powder compositions may be administered, preferably orally or nasally, from devices which deliver the formulation in an appropriate manner.

The following formulation examples illustrate representative pharmaceutical
15 compositions of the present invention.

Formulation Example 1

Hard gelatin capsules containing the following ingredients are prepared:

20	<u>Ingredient</u>	<u>Quantity</u> <u>(mg/capsule)</u>
	Active Ingredient	30.0
	Starch	305.0
	Magnesium stearate	5.0

25

The above ingredients are mixed and filled into hard gelatin capsules in 340 mg quantities.

Formulation Example 2

A tablet formula is prepared using the ingredients below:

	<u>Ingredient</u>	<u>Quantity</u>
		<u>(mg/tablet)</u>
5	Active Ingredient	25.0
	Cellulose, microcrystalline	200.0
	Colloidal silicon dioxide	10.0
	Stearic acid	5.0
	The components are blended and compressed to form tablets, each weighing	
10	240 mg.	

Formulation Example 3

A dry powder inhaler formulation is prepared containing the following components:

	<u>Ingredient</u>	<u>Weight %</u>
15	Active Ingredient	5
	Lactose	95

The active ingredient is mixed with the lactose and the mixture is added to a dry powder inhaling appliance.

Formulation Example 4

Tablets, each containing 30 mg of active ingredient, are prepared as follows:

	<u>Ingredient</u>	<u>Quantity</u>
		<u>(mg/tablet)</u>
25	Active Ingredient	30.0 mg
	Starch	45.0 mg
	Microcrystalline cellulose	35.0 mg
30	Polyvinylpyrrolidone	
	(as 10% solution in sterile water)	4.0 mg
	Sodium carboxymethyl starch	4.5 mg
	Magnesium stearate	0.5 mg
	Talc	<u>1.0 mg</u>
35	Total	120 mg

The active ingredient, starch and cellulose are passed through a No. 20 mesh U.S. sieve and mixed thoroughly. The solution of polyvinylpyrrolidone is mixed with the resultant powders, which are then passed through a 16 mesh U.S. sieve. The granules so produced are dried at 50° to 60°C and passed through a 16 mesh U.S. sieve. The sodium carboxymethyl starch, magnesium stearate, and talc, previously passed through a No. 30 mesh U.S. sieve, are then added to the granules which, after mixing, are compressed on a tablet machine to yield tablets each weighing 120 mg.

Formulation Example 5

Capsules, each containing 40 mg of medicament are made as follows:

<u>Ingredient</u>	<u>Quantity</u> <u>(mg/capsule)</u>
Active Ingredient	40.0 mg
Starch	109.0 mg
Magnesium stearate	1.0 mg
Total	150.0 mg

The active ingredient, starch, and magnesium stearate are blended, passed through a No. 20 mesh U.S. sieve, and filled into hard gelatin capsules in 150 mg quantities.

Formulation Example 6

Suppositories, each containing 25 mg of active ingredient are made as follows:

<u>Ingredient</u>	<u>Amount</u>
Active Ingredient	25 mg
Saturated fatty acid glycerides to	2,000 mg

The active ingredient is passed through a No. 60 mesh U.S. sieve and suspended in the saturated fatty acid glycerides previously melted using the minimum heat necessary. The mixture is then poured into a suppository mold of nominal 2.0 g capacity and allowed to cool.

Formulation Example 7

Suspensions, each containing 50 mg of medicament per 5.0 mL dose are made as follows:

5	<u>Ingredient</u>	<u>Amount</u>
	Active Ingredient	50.0 mg
	Xanthan gum	4.0 mg
	Sodium carboxymethyl cellulose (11%)	
	Microcrystalline cellulose (89%)	50.0 mg
10	Sucrose	1.75 g
	Sodium benzoate	10.0 mg
	Flavor and Color	q.v.
	Purified water to	5.0 mL

15 The active ingredient, sucrose and xanthan gum are blended, passed through a No. 10 mesh U.S. sieve, and then mixed with a previously made solution of the microcrystalline cellulose and sodium carboxymethyl cellulose in water. The sodium benzoate, flavor, and color are diluted with some of the water and added with stirring. Sufficient water is then added to produce the required volume.

20

Formulation Example 8

	<u>Ingredient</u>	<u>Quantity</u> <u>(mg/capsule)</u>
25	Active Ingredient	15.0 mg
	Starch	407.0 mg
	Magnesium stearate	<u>3.0 mg</u>
30	Total	425.0 mg

The active ingredient, starch, and magnesium stearate are blended, passed through a No. 20 mesh U.S. sieve, and filled into hard gelatin capsules in 425.0 mg quantities.

35

Formulation Example 9

A formulation may be prepared as follows:

	<u>Ingredient</u>	<u>Quantity</u>
5	Active Ingredient	5.0 mg
	Corn Oil	1.0 mL

Formulation Example 10

A topical formulation may be prepared as follows:

10	<u>Ingredient</u>	<u>Quantity</u>
	Active Ingredient	1-10 g
	Emulsifying Wax	30 g
15	Liquid Paraffin	20 g
	White Soft Paraffin	to 100 g

The white soft paraffin is heated until molten. The liquid paraffin and emulsifying wax are incorporated and stirred until dissolved. The active ingredient is added and stirring is continued until dispersed. The mixture is then cooled until solid.

Another preferred formulation employed in the methods of the present invention employs transdermal delivery devices ("patches"). Such transdermal patches may be used to provide continuous or discontinuous infusion of the antisense oligonucleotides of the present invention in controlled amounts. The construction and use of transdermal patches for the delivery of pharmaceutical agents is well known in the art. See, for example, U.S. Patent 5,023,252²³, herein incorporated by reference. Such patches may be constructed for continuous, pulsatile, or on demand delivery of pharmaceutical agents.

Another preferred method of delivery involves "shotgun" delivery of the naked antisense oligonucleotides across the dermal layer. The delivery of "naked" antisense oligonucleotides is well known in the art. See, for example, Felgner et al., U.S. Patent No. 5,580,859²⁴. It is contemplated that the antisense oligonucleotides may be packaged in a lipid vesicle before "shotgun" delivery of the antisense oligonucleotide.

Frequently, it will be desirable or necessary to introduce the pharmaceutical composition to the brain, either directly or indirectly. Direct techniques usually involve placement of a drug delivery catheter into the host's ventricular system to bypass the blood-brain barrier. One such implantable delivery system used for the
5 transport of biological factors to specific anatomical regions of the body is described in U.S. Patent 5,011,472²⁵ which is herein incorporated by reference.

Indirect techniques, which are generally preferred, usually involve formulating the compositions to provide for drug latentiation by the conversion of hydrophilic drugs into lipid-soluble drugs. Latentiation is generally achieved through blocking of the
10 hydroxy, carbonyl, sulfate, and primary amine groups present on the drug to render the drug more lipid soluble and amenable to transportation across the blood-brain barrier. Alternatively, the delivery of hydrophilic drugs may be enhanced by intra-arterial infusion of hypertonic solutions which can transiently open the blood-brain barrier.

Other suitable formulations for use in the present invention can be found in
15 *Remington's Pharmaceutical Sciences*²⁶.

The antisense oligonucleotides or the pharmaceutical composition comprising the antisense oligonucleotides may be packaged into convenient kits providing the necessary materials packaged into suitable containers.

20 Utility

The antisense oligonucleotides of the present invention may be used for a variety of purposes. They may be used to inhibit the expression of the ribonucleotide reductase gene in a microorganism, resulting in the inhibition of growth of that microorganism. They may be used to inhibit the expression of the secA gene in a
25 microorganism, resulting in the inhibition of growth of that microorganism. The oligonucleotides may be used as hybridization probes to detect the presence of the ribonucleotide reductase gene or the secA gene in the microorganism. When so used the oligonucleotides may be labeled with a suitable detectable group (a radioisotope, a ligand, another member of a specific binding pair, for example, biotin). The
30 oligonucleotides may also be used to determine the presence of a particular

microorganism in a biological sample. Finally, the oligonucleotides may be used as molecular weight markers.

In order to further illustrate the present invention and advantages thereof, the following specific examples are given but are not meant to limit the scope of the claims
5 in any way.

EXAMPLES

In the examples below, all temperatures are in degrees Celsius (unless otherwise indicated) and all percentages are weight percentages (also unless otherwise indicated).

10 In the examples below, the following abbreviations have the following meanings. If an abbreviation is not defined, it has its generally accepted meaning:

	μM	=	micromolar
	mM	=	millimolar
15	M	=	molar
	ml	=	milliliter
	μl	=	microliter
	mg	=	milligram
	μg	=	microgram
20	IPTG	=	isopropyl- β -D-thiogalactoside
	PAGE	=	polyacrylamide gel electrophoresis
	PVDF	=	polyvinylidene difluoride
	rpm	=	revolutions per minute
	OD	=	optical density
25	CFU	=	colony forming units
	ΔG	=	free energy, a measurement of oligonucleotide duplex stability
	kcal	=	kilocalories

General Methods in Molecular Biology:

Standard molecular biology techniques known in the art and not specifically described were generally followed as in Sambrook et al.¹⁸; Ausubel et al.¹⁹; and Perbal²⁷.

5 The antisense oligonucleotides in Tables 1, 2 and 3 were selected from the sequence complementary to the ribonucleotide reductase or secA genes of *E. coli* such that the sequence exhibited the least likelihood of showing one or more of duplex formation, hair-pin formation, and homooligomer/sequence repeats but had a high to moderate potential to bind to the ribonucleotide reductase gene or the secA gene
10 sequence. These properties were determined using the computer modeling program OLIGO Primer Analysis Software, Version 5.0 (distributed by National Biosciences, Inc., Plymouth, MN).

 The antisense oligonucleotides in Table 4 were selected on the basis that the sequence is highly conserved for the secA genes between two or more microbial
15 species. This property was determined using the BLASTN program (Altschul, et al.¹⁶) of the University of Wisconsin Computer group (GCG) software (Devereux J. et al.¹⁷) with the National Center for Biotechnology Information (NCBI) databases

 Phosphorothioate oligonucleotides comprising the desired sequences were specially ordered either from Boston BioSystems, Bedford MA; Canadian Life
20 Technologies, Burlington, Canada; Dalton Chemical Laboratories, Inc., North York, Canada; Hybridon, Inc., Milford Ma; Oligos Etc., or Oligos Therapeutics, Inc., Wilsonville OR; or TriLink Bio Technologies, San Diego, CA. Antisense oligonucleotides may also be made by methods known in the art.

 Polymerase chain reaction (PCR) was carried out generally as in *PCR*
25 *Protocols: A Guide To Methods And Applications*²⁸.

Example 1: Inhibition of mouse ribonucleotide reductase small subunit (R2) expression in *Escherichia coli* by antisense oligonucleotide AS-II-626-20

Competent BL21 (DE3) cells carrying a plasmid containing the mouse ribonucleotide reductase R2 gene were used. (Mann et al.³⁴) The antisense oligonucleotide, AS-II-626-20, GGCTAAATCGCTCCACCAAG [SEQ ID NO:266] is specifically complementary to the mouse ribonucleotide reductase R2 gene. Approximately 10^{10} bacteria/ml were electroporated using a Cell Porator (Gibco BRL, Burlington, Canada) in micro electro-chambers (0.4 cm between the electrodes) at a pulse of 2.4 kV, 4 k Ω with either 20 μ M or 200 μ M of antisense oligonucleotide AS-II-626-20, following methods described by the manufacturer (Dower W.J.²⁹ ; Neuman et; and Taketo, A.³¹). Control populations were subjected to electroporation but without the antisense oligonucleotide AS-II-626-20.

The bacterial cells were then transferred to Luria-Bertani broth (Miller J.H.³²) containing 50 μ g/ml of ampicillin and 0.4 mM of isopropyl β -D-thiogalactoside (IPTG) (expression inducer) (Horwitz J.P.³³) to grow at 30°C on a shaker at 250 rotations per minute (rpm) for 5 hours.

The cells were harvested by centrifugation and treated with 2 x sample loading buffer (100 mM Tris[hydroxymethyl]aminomethane, pH 6.8, 200 mM dithiothreitol, 4% sodium dodecyl sulfate, 20% glycerol and 0.015% bromophenol blue) and sonicated (Olsvik, et al.³⁵) for 15 seconds. The supernatants were resolved by polyacrylamide gel electrophoresis (PAGE) (Laemmli U.K.³⁶).

The ribonucleotide reductase R2 expression was detected by Western blot. The protein gel was blotted onto polyvinylidene difluoride (PVDF) protein sequencing membrane. (Choy et al.³⁷). The presence of the mouse ribonucleotide reductase was detected with a rabbit anti-mouse R2 subunit antibody (Chan et al.³⁹). The presence of the antibody bound to the ribonucleotide reductase was detected using a second goat anti-rabbit immunoglobulin linked with horseradish peroxidase (Amersham Life Sciences, Oakville Canada).

The upper panel of Figure 14 is a photograph of the Western Blot results. The lower panel of Figure 14 is a photograph of the membrane stained with India ink to indicate the level of protein loaded in each lane.

It is clear that administration of either 20 μ M or 200 μ M AS-II-626-20 resulted in a marked reduction of mouse ribonucleotide reductase gene expression in the *E. coli* cells.

Example 2: Inhibition of bacteria *Escherichia coli* K12 growth by antisense oligonucleotides ER1-169 and ER2-724 targeting *E. coli* ribonucleotide reductase large subunit (R1) and small subunit (R2)

E. coli cells were electroporated by the method set forth in Example 1 with ER1-169 [SEQ ID NO:22] or ER2-724 [SEQ ID NO:145] at the concentrations shown in Figure 15, while the control cells received oligonucleotide AS-II-626-20 [SEQ ID NO:] (targeting mouse ribonucleotide reductase small subunit).

The *E. coli* cells were then transferred to fresh Luria-Bertani broth (Miller J.H.³²) to grow at 30°C on a shaker at 250 rpm for 3 hours. The flasks for the test and the control each contained the same number of bacteria per ml at the start of the experiment. The optical density at 590 nm (OD₅₉₀) of the cultures was measured at the start and at the end of the 3 hours. The inhibition of *E. coli* growth was calculated by comparing the increase in OD₅₉₀ values at the start and the end of the 3 hours of the oligonucleotide-treated cultures to the increase of the control cultures at the start and at the end of the 3 hours. (Carpentier P.L.⁴⁰)

The results indicate that ER1-169 [SEQ ID NO:22] and ER2-724 [SEQ ID NO:145] inhibited the growth of *E. coli*.

Example 3: Killing of *Escherichia coli* K12 by antisense oligonucleotides targeting the ribonucleotide reductase large subunit (R1) or the small subunit (R2)

E. coli cells (approximately 2×10^9) were incubated with 20 μ M of each of the phosphorothioate oligonucleotides set forth in Figure 12 on ice for 45 minutes. A

control without oligonucleotides was also incubated for each experiment. Cells were heat shocked by placing them in a 42°C bath for 45 seconds. (Sambrook J. et al.¹⁸)

Luria-Bertani (LB) broth (Miller J.H.³²) was added and the samples were incubated at room temperature for 30 minutes. Dilutions of treated and untreated bacteria were incubated overnight at 37°C on culture plates containing LB medium, and the number of colonies was counted.

The number of killed bacteria was calculated by subtracting the surviving colony forming units (CFU/ml) of the oligonucleotide-treated bacteria from the CFU/ml of the control. Figure 16 shows the number of bacteria killed by treatment with the antisense sequences: ER1-640 [SEQ ID NO:43]; ER1-1059 [SEQ ID NO:62]; ER1-1320 [SEQ ID NO:75]; ER1-1315 [SEQ ID NO:74]; ER1-1326 [SEQ ID NO:76]; ER2-704 [SEQ ID NO:143] and ER2-983 [SEQ ID NO:152].

The results from Figure 16 show that antisense oligonucleotides complementary to either the R1 or R2 subunit of ribonucleotide reductase are effective as anti-bacterial agents.

Example 4: Inhibition of the secA protein expression in Escherichia coli following treatment with antisense phosphorothioate oligonucleotides

E. coli cells were heat shock transformed by the method set forth in Example 3 above with the 80 µM of each of the antisense phosphorothioate oligonucleotides set forth in Figure 17.

Luria-Bertani broth was then added to the treated *E. coli* cells and they were allowed to grow at 30°C on a shaker at 250 rpm for 3 hours.

Approximately the same quantity of treated and untreated bacteria, based on optical density, were washed in phosphate buffered saline, suspended in 2X Laemmli sample buffer (Laemmli U.K.³⁶), heated for 5 minutes at 95°C and subjected to SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis).

The gel was blotted onto polyvinylidene difluoride protein sequencing membrane by the methods set forth in Example 1. A rabbit polyclonal SecA antiserum (der Blaauwen et al.⁶) was used to detect the expression of the *E. coli* secA gene. The presence of bound rabbit antibody was detected using a goat anti-rabbit immunoglobulin (Amersham Life Sciences, Oakville, Canada).

Figure 17 is a photograph of the Western Blot of *E. coli* cells treated with oligonucleotides ES799 [SEQ ID NO:195] (lane 1); ES1845 [SEQ ID NO:235] (lane 2); and the control (lane 3). When compared to the control, lane 3, the ES799 [SEQ ID NO:195] and ES1845 [SEQ ID NO:235] oligonucleotides clearly decreased the SecA protein levels in the treated *E. coli* cells. The top band in the Figure 17 represents SecA. Non-specific background bands appear below the SecA protein band.

It has been found that the antisense oligonucleotides are effective inhibitors of SecA expression in *E. coli*.

15 Example 5: Killing of Escherichia coli K12 by antisense secA oligonucleotides

E. coli cells were heat shock transformed by the method described in Example 3 above with either 100 μ M or 20 μ M of the antisense phosphorothioate oligonucleotides set forth in Figures 18a and 18b

Luria-Bertani (LB) broth (Miller J.H.³²) was added and the bacterial samples were incubated at room temperature for 30 minutes. Dilutions of treated and untreated bacteria were incubated overnight at 37°C on culture plates containing LB medium, and the number of colonies was counted.

The number of killed bacteria was calculated by subtracting the surviving colony forming units (CFU/ml) of the oligonucleotide-treated bacteria from the CFU/ml of the control. Figures 18a and 18b show the number of bacteria killed by treatment with the various antisense sequences. Accordingly, antisense oligonucleotides complementary to the secA gene act to inhibit the growth of *E. coli*.

Example 6: Effect of antisense oligonucleotides on Escherichia coli K12 growth

E. coli cells were heat shock transformed by the method described in Example 3 with either 16 μ M, 20 μ M or 80 μ M of each of the antisense phosphorothioate oligonucleotides set forth in Figures 19a-g.

5 Equal numbers of the treated *E. coli* cells were then transferred to flasks containing fresh Luria-Bertani broth to grow at 30°C on a shaker at 250 rpm. The number of bacteria per flask was determined by the turbidity of the cultures at OD₆₂₀ taken each hour (Carpentier P.L.⁴⁰).

10 Figures 19a-g show the rate of growth of the *E. coli* in each of the flasks after treatment with the various oligonucleotides. When growth curves of the treated and untreated cultures were statistically analyzed, the growth of the antisense treated cultures was found to be significantly inhibited when compared to the control cultures. The statistical p values are found in the Figures.

Claims:

1. An antisense oligonucleotide which is nuclease resistant and comprises from about 3 to about 50 nucleotides, which nucleotides are complementary to the ribonucleotide reductase gene or the secA gene of a microorganism.
- 5 2. The oligonucleotide of Claim 1 comprising one or more phosphorothioate internucleotide linkages.
3. An antisense oligonucleotide comprising from about 3 to about 50
10 nucleotides which is capable of binding to the ribonucleotide reductase gene or the secA gene of a microorganism, wherein the oligonucleotide comprises all or part of a sequence selected from the group consisting of SEQ ID NO:22; SEQ ID NO:43; SEQ ID NO:62; SEQ ID NO:74; SEQ ID NO:75; SEQ ID NO:76; SEQ ID NO:143; SEQ ID NO:145; SEQ ID NO:152; SEQ ID NO:164; SEQ ID NO:176; SEQ ID NO:186;
15 SEQ ID NO:188; SEQ ID NO:189; SEQ ID NO:191; SEQ ID NO:192; SEQ ID NO:195; SEQ ID NO:197; SEQ ID NO:206; SEQ ID NO:212; SEQ ID NO:220; SEQ ID NO:229; SEQ ID NO:235; SEQ ID NO:254; SEQ ID NO:261; SEQ ID NO:262; SEQ ID NO:263; SEQ ID NO:264; and SEQ ID NO:265.
- 20 4. A pharmaceutical composition comprising a pharmaceutically acceptable excipient and an effective amount of an oligonucleotide which is nuclease resistant and comprises from about 3 to about 50 nucleotides, which nucleotides are complementary to the ribonucleotide reductase gene or the secA gene of a microorganism.
- 25 5. The pharmaceutical composition comprising a pharmaceutically acceptable excipient and an effective amount of an oligonucleotide comprising from about 3 to about 50 nucleotides which is capable of binding to the ribonucleotide reductase gene or the secA gene of a microorganism, wherein the oligonucleotide comprises all or part of a sequence selected from the group consisting of SEQ ID NO:22; SEQ ID NO:43;
30 SEQ ID NO:62; SEQ ID NO:74; SEQ ID NO:75; SEQ ID NO:76; SEQ ID NO:143;

SEQ ID NO:145; SEQ ID NO:152; SEQ ID NO:164; SEQ ID NO:176; SEQ ID NO:186; SEQ ID NO:188; SEQ ID NO:189; SEQ ID NO:191; SEQ ID NO:192; SEQ ID NO:195; SEQ ID NO:197; SEQ ID NO:206; SEQ ID NO:212; SEQ ID NO:220; SEQ ID NO:229; SEQ ID NO:235; SEQ ID NO:254; SEQ ID NO:261; SEQ ID NO:262; SEQ ID NO:263; SEQ ID NO:264; and SEQ ID NO:265.

6. A method of inhibiting the expression of a ribonucleotide reductase gene in a microorganism having a ribonucleotide reductase gene, comprising administering to said microorganism or to a cell infected with said microorganism an effective amount of an antisense oligonucleotide comprising from at least about 3 nucleotides which are complementary to the ribonucleotide reductase gene of the microorganism under conditions such that the expression of the ribonucleotide reductase gene is inhibited.

7. The method according to Claim 6, wherein said microorganism is a bacterial cell.

8. The method according to Claim 6, wherein said microorganism is a virus.

9. The method according to Claim 6 wherein the antisense oligonucleotide comprises a sequence selected from the group consisting of SEQ ID NO:22; SEQ ID NO:43; SEQ ID NO:62; SEQ ID NO:74; SEQ ID NO:75; SEQ ID NO:76; SEQ ID NO:143; SEQ ID NO:145; and SEQ ID NO:152.

10. A method of inhibiting the expression of the secA gene in a microorganism having a secA gene, comprising administering to said microorganism an effective amount of an antisense oligonucleotide comprising from at least about 3 nucleotides which are complementary to the secA gene of the microorganism under conditions such that the secA gene is inhibited.

11. The method according to Claim 10, wherein said microorganism is a bacterial cell.

12. The method according to Claim 11 wherein the antisense oligonucleotide
5 comprises a sequence selected from the group consisting of SEQ ID NO:164; SEQ ID
NO:176; SEQ ID NO:186; SEQ ID NO:188; SEQ ID NO:189; SEQ ID NO:191; SEQ
ID NO:192; SEQ ID NO:195; SEQ ID NO:197; SEQ ID NO:206; SEQ ID NO:212;
SEQ ID NO:220; SEQ ID NO:229; SEQ ID NO:235; SEQ ID NO:254; SEQ ID
NO:261; SEQ ID NO:262; SEQ ID NO:263; SEQ ID NO:264; and SEQ ID NO:265.

10

13. A method of inhibiting the growth of a microorganism having a
ribonucleotide reductase gene or a secA gene, which method comprises identifying the
microorganism and administering to said microorganism an effective amount of an
antisense oligonucleotide comprising from at least about 3 nucleotides which are
15 complementary to either the ribonucleotide reductase gene or the secA gene of the
microorganism under conditions whereby the growth of the microorganism is inhibited.

14. The method according to Claim 13, wherein said microorganism is a
bacterial cell.

20

15. The method according to Claim 13, wherein said microorganism is a virus.

16. The method according to Claim 13 wherein the antisense oligonucleotide
comprises a sequence selected from the group consisting of SEQ ID NO:22; SEQ ID
25 NO:43; SEQ ID NO:62; SEQ ID NO:74; SEQ ID NO:75; SEQ ID NO:76; SEQ ID
NO:143; SEQ ID NO:145; SEQ ID NO:152; SEQ ID NO:164; SEQ ID NO:176; SEQ
ID NO:186; SEQ ID NO:188; SEQ ID NO:189; SEQ ID NO:191; SEQ ID NO:192;
SEQ ID NO:195; SEQ ID NO:197; SEQ ID NO:206; SEQ ID NO:212; SEQ ID
NO:220; SEQ ID NO:229; SEQ ID NO:235; SEQ ID NO:254; SEQ ID NO:261; SEQ
30 ID NO:262; SEQ ID NO:263; SEQ ID NO:264; and SEQ ID NO:265.

17. A method for treating a mammalian pathologic condition mediated by microorganisms, which method comprises identifying a mammal having a pathologic condition mediated by microorganisms having a ribonucleotide reductase gene or a secA gene and administering to said mammal an effective amount of an antisense
- 5 oligonucleotide comprising at least about 3 nucleotides which are complementary to either the ribonucleotide reductase gene or the secA gene of the microorganism under conditions such that the growth of the microorganism is inhibited.

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1 atgaatcaga atctgctgggt gacaaagcgc gacggtagca cagagcgcgt caatctcgac
61 aaaatccatc gcgttcttga ttgggcggca gaaggactgc ataacgtttc gatttcccag
121 gtcgagctgc gctcccacat tcagttttat gacggtatca agacctctga catccacgaa
181 accattatca aggetgccgc agacctgac tcacctgagt ccccgtagt cagtatctc
241 gccgcgcgcc tggcgatctt ccacctgcgt aaaaagcct acggccagtt tgagccgct
301 gcgctgtacg accacgtggt gaaaatggtc gagatggga aatcagataa tcactgtctg
361 gaagactaca cgggaagaaga gttcaagcag atggacacct ttatcgatca cgaccgtgat
421 atgaccttct cttatgctgc cgttaagcag ctggaaggca aatatctggt acagaaccgc
481 gtgaccggcg aatatctatga ggcgcccag ttctttata ttctagttgc cgcgtgcttg
541 ttctcgaaet acccgcgtag aacgcgcctg caatatgtga agcgttttta cgacgcggtt
601 tccacattta aatttctgct gccgacgcca atcatgtccg gcgtgcgtac cccgactcgt
661 cagttcagct cctgcgtact gctcgagtgc ggtgacagcc tggattccat caacgccacc
721 tccagcgcga ttgttaata cgtttcccag cgtgcggga tcggcataca cgccgggcgt
781 attcgtgcgc tgggtagccc gattcgcggt ggtgaagcgt tccataccgg ctgcatlccg
841 ttctacaaac atttccagac agcggtagaa tctgtctctc agggcgggtt gcgcggcgggt
901 gcggcaacgc tgttctaccc gatgtggcat ctggaagtgg aaagccctgct ggtgttgaaa
961 aacaaccgtg gtgtggaagg caaccgcgtg cgtcatatgg actacggggt acaaatcaac
1021 aactgatgt ataccgtct gctgaagggt gaagatataa cctgttccag cccgtccgac
1081 gtaccggggc tgtaccgacgc gttcttcgcc gatcagggaag agtttgaacg tctgtatccc
1141 aatatatgaga aagacgacag catccgcaag cagcgtgtga aagccgttga gctgttctcg
1201 ctgatgatgc aggaacgtgc gttaccgggt cgtatctata ttcagaacgt tgaccactgc
1261 aatacccata gcccgtttga tccggccatc gcgccagtgc gtcagtctaa cctgtgcctg
1321 gagatagccc tgcgaccaca accgctgaac gacgtcaacg acgagaacgg tgaatcgcg

FIG. 1A

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1381 ctgtgtacgc tgtctgcttt caacctgggc gcaattaata acctggatga actggaagag
1441 ctggcaattc tggcggttcg tgcacttgac gcgctgctgg attatcagga ttacccgatc
1501 ccggccgcc aacgtggagc gatgggtcgt cgtacgctgg gtattggtgt gateaacttc
1561 gcttactacc tggcgaaacga cggtaaacgc tactccgacg gcagcgccaa caacctgacg
1621 cataaaacct tcgaagccat tcagtattac ctgctgaag cctctaata gctggcgaaa
1681 gagcaaggcg cgtgccctg gtttaacgaa accacttac cgaagggat cctgccgac
1741 gatacctata agaagatct ggataccatc gctaattgagc cgctgcattt cgactgggaa
1801 gctctgcgtg agtcaatcaa aacgcacggc ctgcgtaact ccacgcttcc tgcctctgatg
1861 ccgtccgaga cttcttcgca gatctctaac gccactaacg gtattgaacc gccgcgcgg
1921 tacgtcagca tcaaacgctc gaaagacggc attttgcgcc aggtggtgcc ggactacgag
1981 cacctgcacg acgcctatga gctgctgtgg gaaatgccgg gtaacgatgg ttatcttgc
2041 ctggtgggta tcatgcagaa atttategat cagtcgatct ctgccaacac caactacgat
2101 ccgtcacgct tccgctcagg aaaagtgcg atgcagcagt tgctgaaaga cctgctcdcc
2161 gcctacaaat tcgggggtcaa aacactgtat tatcagaaca ccgctgacgg cgctgaagac
2221 gcacaagacg atctggtgcc gtcaatccag gacgatggc gcgaaagcgg cgcatgtaag
2281 atctga

FIG. 1B

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7381 ctggtgccgt caatccagga cgaagggtgc gaaagcggcg catgtaagat ctgatattga
 7441 gatgccggat gcggcgtaaa cgcctatacc ggcctacggc tcggtttgta ggccgtgataa
 7501 gacgcgccag cgtcgcatca ggctccgggt gccggatgca gcgtgaacgc cttatccggc
 7561 ctacggctcg gatttgtagg cctgataaga cgcgccagcg tcgcatcagg cacaggatgc
 7621 ggcgtaaat gccttatccg gcattaaact cccaacagga cacactcatg gcataacca
 7681 ccttttca gacgaaaaat gatcagctca aagaaccgat gttctttggt cagccggta
 7741 acgtggctcg ctacgatcag caaaaatatg acatcttcca aagctgac gaaaagcagc
 7801 tctctttctt ctggcgctcg gaagaagtgg acgtctcccg cgaccgtata gattaccagg
 7861 cgtgcccgga gacgaaaaa cacatcttta tcagcaacct gaaatatacag acgtgtctgg
 7921 attccattca gggtcgtagc ccgaacgtgg cgtattgcc gcttattct attccggaac
 7981 tggaaacctg ggtcgaaacc tgggcgttct cagaaacgat tcattcccg tctatactc
 8041 atatcattcg taatatcggt aacgatccgt ctggtgtgtt tgacgatata gtcaccaacg
 8101 agcagatcca gaaacgtgcg gaagggatct ccagctatta cgatgagctg atcgaaatga
 8161 ccagctactg gcattctgctg ggcgaaggta cccacaccgt taacggtaaa actgtgaccg
 8221 tttagcctgcg cgagctgaag aaaaaactgt atctctgcct gatgagcgtt aacgcgtgg
 8281 aagcgattcg tttctacgta agctttgctt gttccttcgc atttgcagaa cgcgaattga
 8341 tggaaaggcaa cgccaaaatt attcgctga ttgccccgca cgaagccctg cacctgaccg

FIG. 2A

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8401 gcacccagca tatgtgaat ctgtgcgca gcggcgcgga cgatcctgag atggcggaaa
8461 ttgcgaaga gtgtaagcag gagtgtctatg acctgtttgt tcaggcagct caacaggaga
8521 aagactgggc ggatttatctg ttccgcgacg gttcgatgat tggcttgat aaagacattc
8581 tctgccagta cgttgaatc atcaccata tccgtatgca ggcagtcggt ttggatctgc
8641 cgttccagac gcgtccaac ccgataccgt ggatcaaac ttggttggtg tctgataacg
8701 tgcaggttgc tccgcaggaa gtggaagtca gttcttatct ggtcgggcag attgactcgg
8761 aagtggacac cgacgatctg agtaacttcc agctctgatg gcccgcgta cctgcgcac
8821 cactggcaca caactgtgt gccaggatga acaccttcc ctcttggcgg cgtgggaatc
8881 ccacaatgtg gcggttgagt accagtgtcg cgaaggttac tgcggctcct gtcgcacacg

FIG. 2B

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301 gtgaacgtcg atctggtgcc ggatgcagcg gatacgetcc gggcgcaagg atttcgtcaa
 361 ttaccggtgg tgatggcggg cgatttgagc tggctctggct tccgcccggg catgattaac
 421 cgtctgcacc cgacacccca cgcggcaaac gcatgagcgc gctcgtctac ttctccagca
 481 gctctgaaaa tacgcaccgc tttatgcagc gtctggggtt gctgcccacg cgtattccgc
 541 tcaatgagcg ggagcgaatt caggtagacg aaccgtacat tctggttggt ccgctcatac
 601 gcggcgccgg gatggccggt gcggtgccgc gacaggtgat ccgcttttta aatgatgaac
 661 acaaccgggc gcgcatcgc ggcattcgc ggcgttatcg cctccggtaa tcgcaatttc ggcgatgcct
 721 ggggatgcgc tggcgatgtg atagcacaaa aatgcggcgt cccctggctg taccgctttg
 781 agctcatggg cacacaacgc gacatcgata atgtccgaaa aggagtaaat gaattttggc
 841 acaactacc cggagcgcg taatgcagga aaccatggat taccacgccc tgaacgcgat
 901 gctgaatctt tacgataaag caggccatat tcagttcgac agggaccacg aggcgatcga
 961 cgccttcttt gccaccacag tccgcccga ttccgtgacg tttgccagcc agcatgaacg
 1021 tetggggacg ctggttcggg aagggtatta cgatgacgc gtcctcgcgc gttacgacccg
 1081 cgccttcgtc cttegcctgt tcgagcacgc ccatgccagc ggctttcgct tccagacgtt
 1141 tcttggcgc tggagttct ataccagtta cagctgaaa accctcgacg gcaaacgtta
 1201 tctggaacac tttgaagatc ggtgacaat ggtggcgttg acgctggcgc aggtgaéga
 1261 aacgctggcc acccaactga ccgatgaaat gctttctggt cgttttcagc ccgctacccc
 1321 gactttttta aattgcggca aacagcagcg tggggaaactg gtctcctgct tctgctccg
 1381 tategaagac aacatggagt cgatcgggcg ggcggtgaat tcggcgctgc aactctccaa
 1441 acgcgccggc ggcgtcgcgt ttttactctc caatctgcgc gaggcggcg cgccgatcaa
 1501 acgcattgag aatcagttt ccggcgtgat cccggtgatg aaaatgctgg aagacgcgtt
 1561 ttcgtatgcc aaccaacttg gcgcgcgcca gggggccggc gcggtttatc tccatgcga
 1621 ccatccggat attctgcgtt ttctggatac caacgagaa aacgctgacg aaaaaatccg

FIG. 3A

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1681 gatcaaaacg ctctctctcg gcgtggtgat cccggatata accttcggc tggcgaaaga
1741 aaacgcgcaa atggcgctct tttegccta tgacatacaa cgacgctacg geaaaccgtt
1801 tggcgatac gccattagcg aacggtaacga tgaattaat gccgatccgc acgtgcgcaa
1861 aacctatatt aacgcccgtg acctttttca aacactggcg gagattcagt tcgaatccgg
1921 gtatccctac atcatgtttg aagatacggc aaaccgcgcg atccccattg ctggtcgcac
1981 taatatgagc aacctgtgct cagaaatttt acaggccaat agcgttccc gttacgacga
2041 taaccttgac tatacccaca tcgggcatga catctcctgc aatctcggct cgctgaatat
2101 cgctcacgct atggattcac cggocattgg ccgtaccgta gaaccgcta ttccgcggcct
2161 gacggcggtg tcggacatga gccatatocg cagcgtgcc tcaatagccg ccggtaatgc
2221 cgctctcat gccatcggtc tgggccagat gaatctgcat ggctatctgg cgagggaagg
2281 tattgcctac gtttcgccgg aggcgttggg tttaaccaat ctctattttt acaccattac
2341 ctggcatgcc gtgcatactt caatgcggct agccgcgaa cgcggaacaa ccttcgccgg
2401 atttgcgcag tcgcgctatg ccagcggcga ctattttac cagtatttac aggcgcactg
2461 gcaaccgaaa acagcgaaag tcagggcgtt atttgccgc agcgcatcta cgctgccac
2521 acgagaaatg tgctaaagc tgcgcgaacga tgtgatgccc tatggcatct ataaccacaa
2581 tttagcaggcg gtgccgccga ccggttcgat ttcttacatt aatcatgcca cctccagcat
2641 tcatccgatt gtgcccacaa ttgagattcg caaagagggc aaaccgggc gtgtgtatta
2701 cccgcgcgcg tttatgacca atgaaaacct ggacatgtat caggatgctt acgatatcgg
2761 tccggaaaaa attattgata cctatgccga gcccacgcgc cactcgatc aaggctgtc
2821 gctacccctg tttttcccg ataccgccac gaccgcgat atcaacaagg cgcagatcta
2881 tgcctggcga aaaggtatta agtccctgta ttacatcccg cttcgccagt tggcgctgga
2941 aggtactgaa attgaaggct gcgtatcctg cgcgctataa ggaagccat atgaattat
3001 ctcgtattag cgccatcaac tggacaaga tccaggacga caagatctg gaggatatgga

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FIG. 3B

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3061 accggtgac cagtaacttc tggtgcggg aaaaagtgcc gttatcgat gatttccgg
 3121 cctggcagac gctgagcgcc gccgaacagc agctcaccat tcgctgttt acgggactta
 3181 cgtgctcga cactatccag aacatgcgag gcgcgcgctc gttaatggca gatgccatca
 3241 cgccgcagtga agaggcagtg ctgtcgaaac tcagctttat ggaagcggta caagcccgct
 3301 cttacagttc tattttctcc acgctgtgcc agcggaaaga ggttgatgcc gcctacgcct
 3361 ggagcgaaga aaaccaccgc cttcagcgta aggcgcagat tattttagct cattacgtca
 3421 gcatgaacc gctaaagaaa aagattgcca gcgtcttttt agagtccttt ctgttctatt
 3481 cggcttctg gttgcegatg tatttctcca gccgcggtaa gctcacgaac actgccgacc
 3541 tgattcgttt aatcattcgc gatgaagcgg ttccacggta ttatatggc tataagtatc
 3601 agatagcgt acaaaaaacta tcggcaatcg agcgtgaaga gttaaagctt ttgcgcgtgg
 3661 atttgttgat ggaactgtac gacaacgaaa tccgctacac agaagcgtta tatgcggaaa
 3721 ccggttgggt taacgacgct aaagccttct tggtctacaa cgccaataaa gccttaatga
 3781 acctggttta tgaggcgtta ttccgcggg agatggcaga cgtgaatccc gcaatccttg
 3841 ccgcgctctc gccgaatgcc gacgaaaacc atgatttctt ttccggctca ggttcactctt
 3901 atgtgatggg gaaacacgct gaaaccgaag acgaagactg gaatttttaa ccttacgggc
 3961 atgggaaata acgttacatt tcccatgcct ttattttcaag caatagggag tcaaatcgcg
 4021 caaatattac aacatgtcct acactcaata cgagtgcacat tattcacctg gattccccca
 4081 attcaggctg atttttgctg gttgttccaa aaaatatctc ttccctcccca ttccgcgttca
 4141 gcccttatat catgggaaat cacagccgat agcacctcgc aatattcatg ccagaagcaa
 4201 attcagggtt gtctcagatt ctgagtatgt tagggtagaa aaaggtaact atttctatca
 4261 ggtaacatat cgacataagt aaataacagg aatcattcta ttgcatggca attaaattag
 4321 aagtgaagaa tctgtataaa atatttggag agcatccgca gcgtgccttc aaatatattg
 4381 aaaagggact atcgaagag caaatactgg aaaaaacggg gctatcgctt ggcgttaaag

FIG. 3C

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4441 acgccagtct ggccattgaa gaaggcgaga tatttgtcat catgggatta tccggctcgg
4501 gtaaatccac aatggtacgc cttctcaate gctgattga acccaaccgc ggacaggtag
4561 tgattgacgg cgttgatatt gccaaatat cagacgctga gcttcgcgag gtgcgcagga
4621 aaaagattgc gatggtcttc cagtcatttg cgtcatgcc gcataatgacc gtgctggata
4681 atacggcatt cggatatgaa tttagcgggca tcgcggcgca agagcgtgc gaaaaagcgc
4741 tggacgccct gcgtcagggtg gggcttgaga attacgctca cgcctacccg gatgaacttt
4801 ccggtgggat gcgtcagcgt gttgggcttg cccgcgcgct ggcaatcaac cctgatatct
4861 tattaatgga tgaagcggtt tccgccctcg atcc

FIG. 3D

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1 gaatttcttat ttccctagc ttggattta ttctcacttc ctatgatctt ttattctcga
61 ttattatttt tgccttgga attattatca tttttcgaca taaacacaaac ctcaaaagaa
121 tcaaaaatca ttgtgaatcc cttgtccctt ttggtttaaa ctatcagaga caaaaagaaa
181 aatageacaa tatatttgtt tgttttctt tttttacata atttaacact atatctagta
241 tctttaattt gactagatat tttttttacg ctaataaga ctataaaaac tcgagaaaaa
301 gtcaaggact ttttaactcc gtctaaaaa tatattggcc caaaaggaga tttaaaatgg
361 ttacagttta ttctaaaaa aattgtatgc aatgcaaaat ggtaaaaaa tggctttctg
421 aacacgaat tgcatttaac gaaatcaata ttgotgaaca gctgaattt gtcgaaaaag
481 taattgaaat gggttttcga gctgctcctg taatcacaaa agatgatttc gccttttctg
541 gtttccgtcc ttctgaatta gcaagttgg cttaatatga aacttgctta tttcagtggtg
601 actggacaaa cgcgtcgttt tgtttctaaa acagacttgc cgaatgtcga aattacacct
661 gacgatgatt tagagatgga cgagccttcc cttttgataa ctccctctta tgcgtgaaga
721 tcaccaaccg tttctaaatc aatagacgtt atggactcgg tttttgactt tatggcttat
781 aatgataatt ataacatttg tcgtggaatt atcggcaactg gaaatcgtaa ttttgcgtgc
841 atctatatat ttaccgctaa agaagtttca gcaaatatc aaattccact tttatatgat
901 tttgagttta atggtacgcc agctgatgtt gctgctgttg aaaaactcgc tgcacagctt
961 gatcaaggag cgaagtcac ctttaaaaat ccgtgtgat tttttatggc ttcaacctat
1021 ttgagtgaag ctt

```

FIG. 4

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1 cagctgtact ggcataacga catttatact gtcgtataaa attcgactgg
51 caaatctggc actctctccg gccaggtaga ccagtcgttt ttttttgat
101 tttataagag ctataaaaa cggtagcgaac gctgttttct taagcacttt
151 tccgcacaac ttatcttcat tcgtgctgtg gactgcaggc tttaatgata
201 agatttgtgc gctaaatacg tttgaatatg atcgggatgg caataacgtg
251 agtggaaatc tgacgcgtg gcgacagttt ggtaaacgct actctggcc
301 gcatctctta ttagggatgg ttgcggcgag tttagggttg cctgcgctca
351 gcaacgcgc cgaaccaaac gcgccgcaa aagcgacaac ccgcaaccac
401 gagccttcag ccaagttaa ctttggtcaa ttggccttgc tggaaagcgaa
451 cacacgcgc cgaattcga actattccgt tgattactgg catcaacatg
501 ccattcgcac gtaatccgt catctttctt tcgcaatggc accgcaacaa
551 ctgcccgttg ctgaagaatc ttgacctt caggcgcaac atcttgcat
601 actggatacg ctacgcgcgc tgctgacca ggaaggcacg ccgtctgaaa
651 agggttatcg cattgattat gcgcatttta cccacaagc aaatttcagc
701 acgcccgtct ggataagcca ggcgaaggc atccgtgctg gccctcaacg
751 cctcacctaa caacaataaa cctttacttc attttattaa ctccgcaacg
801 cggggcgttt gagattttat tatgctaalc aaattgttaa ctaaagtttt
851 cagtagtcgt aacgategca cctgcgcgc gatgcgcaa gtggtcaaca
901 tcatcaatgc catggaaccg gagatggaaa aactctccga cgaagaactg
951 aaaaqgaaaa ccacagagtt tcgtcacgt ctggaaaaaa gcgaagtgt
1001 gaaaaatctg atcccqaaa ctttcgcctt ggtacgtgag gcaagtaagc
1051 gcgtcttttg tatgcgtcac ttcqacgttc agttactcgg cgttatggtt
1101 cttaacgaac gctgcctcgc cgaatgcgt accggtgaag gaaaaccct

FIG. 5A

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1151 gaccgcgaacc ctccctccctt accctgaacgc actaaccggt aagggcgtgc
1201 acgtagtattac cgtcaacgac tacctggcgc aacgtgacgc cgaatacaaac
1251 cgcccgctgt ttgaattcct tggcctgact gtcggtatca acctgccggg
1301 catgcacga cggcaaacgc gcgaagctta cgcagctgac atcacttacg
1351 gtaccaacaa cgaatacggc ttltgactacc tgcgcgacaa catggcgttc
1401 agccctgaag aacgtgtaca gcgtaaactg cactatgcgc tggtaggacga
1451 agtggactec atcctgateg atgaagcgcg tacaccgctg atcatttccg
1501 gcccggcaga agacagctcg gaattgtata aacgcgtgaa taaatttatt
1551 cgcacactga tccgtcagga aaaaagaagac tccgaacct tccagggcga
1601 agggcacttc tggatggacg aaaaatctcg ccagggtgaac ctgaccgaac
1651 gtggtctggt gctgattgaa gaactgctgg tgaagaaggc catcatggat
1701 gaaggggagt ctctgtactc tccggccaac atcatgctga tgcaccacgt
1751 aacggcggcg ctgcgcctc atgcctgttt taccctgac gtcgactaca
1801 tcgttaaga tggtagagtt atcctcgttg acgaacacac ccgtcgtacc
1851 atgcagggcc gtcctggtc cgtatggtctg caccaggctg tgaagcga
1901 agaagggtg cagatccaga acgaatacca aacgctggct tgcataccct
1951 tccagaacta ctccgtctg tatgaataac tggcggggat gaccggtact
2001 gctgataccg aagctttcga atttagctca atctacaagc tggataccgt
2051 cgltgttccg accaacctc caatgattcg taagatctg ccggaccctg
2101 tctacatgac tgaagcggaa aaaaattcagg cgtcattga agatatcaaa
2151 gaacgtactg cgaagaagcca gccggtgctg gtgggtacta tctccatcga
2201 aaaatcggag ctggtatcaa acgaactgac caagccggt attaagcaca
2251 acgtcctgaa ccccaattc cagcccaacg aagcggcgat tgttgcctcag

FIG. 5B

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2301 gcagggttate cggctgcggt gactatcgcg accaatatgg cgggtcgtgg
2351 tacagatatt gtgctcggtg ataactggca ggcagaagtt gccgcgctgg
2401 aaatccgac cgcagagcaa attgaaaaaa ttaagccga ctggcaggta
2451 cgtcacgatg cgttactgga agcaggtagc ctgcataatc tccgtaccga
2501 gcgtcacgaa tcccgtcgta tcgataacca gttgcgcggt cattctggtc
2551 gtcaggggga tctgtgttct tcccgtttct acctgtcgat gaaagatgcg
2601 ctgatgcgta tttttgcttc cgaccgagta tccggcatga tccgtaaact
2651 ggttatgaag ccaggcggaag ccattgaaca cccgtgggtg actaaagcga
2701 ttgccaaacg ccagcgtaaa gttgaaagcc gtaacttcga cattcgttaag
2751 caactgctgg aatatgatga cgtggctaac gatacgcgtc gcgccattta
2801 ctcccagcgt aacgaactgt tggatgtcag cgaatgagc gaaaccattta
2851 acagcatttcg tgaagatgtg ttcaagcga ccattgatgc ctacattcca
2901 ccacagtcgc tgaagaaat gtggatatatt ccggggtgc aggaacgtct
2951 gaaqaacgat ttcgacctcg atttgccaat tgcgagtag ctggataaag
3001 aaccagaaact gcataagag acgctgcgtg accgcattct gccgcagtc
3051 atcgaagtgt atcagcgtaa agaaagagt gttgatgctg agatgatgcg
3101 tcacttcgag aaagcgcgtc tctgcgaac gcttgactcc ctgtggaag
3151 agcacctgac agcgatggac tatctgcgtc aggtatacca cctgcgtgac
3201 tacgcacaga aagatccgaa gcaggaaatc aaacgtgaat cgttctccat
3251 gtttgacgcg atgctggagt cgttgaata tgaagttatc agtacgctga
3301 gcaaaattca ggtacgtatg cctgaagagg ttgaggagct ggaacacag
3351 cgtcgtatgg aagccaaacg tttagcgcga atgcagcagc ttagccatca
3401 ggtgacgac tctgcagccg cagctgcact ggcggcgcaa accggagagc

FIG. 5C

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3451 gcacaaatagc acgtaacgat ccttgcccg gcggttctgg taacaaatac
3501 aagcagtacc atggccgcct gcaataaaag ctaactgttg aagtaaaagg
3551 cgcaggattc tgcgcctttt ttataggttt aagacaatga aaagctgca
3601 aattgcggtg ggtattattc gcaacgagaa caatgaaatc ttataacgc
3651 gtcgcgcagc agatgcgcac atggcgata aactggagtt tcccggcgg
3701 aaaattgaaa tgggtgaac gccggaacag gcggtggtgc gtgaacttca
3751 ggaagaagtc gggattaccc cccaacattt ttcgctattt gaaaaactgg
3801 aatatgaatt c

FIG. 5D

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1 gatctacggc agaactcgtc gcttgaggcg ttgcaccgac catctaccctg
51 ttgcacgtcg aactcgacca ctgaacgtaa tcgcgcgcag cgcaagtccct
101 gtcagcgcgt ggagatcacc gcgcgtgggc gagggcgcgt ggtgcgaggt
151 gaggcctgcg ccgacagctt ctatgcgcg cttgaatcag cggtcgtcaa
201 actggagagc gtgcgcgcg gtaaggatcg ccgcaaggctg cactacggcg
251 acaaaacccc ggttcgctg gccgaggcga ccgcggtggt gccagcgccg
301 gagaacggct tcaacaccag accagccgag gcacacgac acgacggctgc
351 cgtcgtcgag cgggagcctg ggcggatcgt tcgcaccaa gaacaccccg
401 ccaagccgat gtcggtcgat gacgcgtct accagatgga gctggttga
451 cagcacttct tcttgtteta cgacaaggac accgaacggc cgtcgggtggt
501 ctaccgccgg cagcctacg actacggctt gatccgtctg gcgtgatcgg
551 cggcgcgcgc cgctcgtcac ctaccatggg agtcgcctta tctaaagact
601 cctacacatg cggggacata gctgtgctgt cgaagtgtct gcgccttggc
651 gaaggtcgca tggteaagcg cctcaagaag gtggcggact atgtcggcac
701 tttgtccgac gatgtcgaga aactcacga cgcggagctg agggcgaaaa
751 ccgacgagtt caagcggcgg ctggccgacc agaaaaaccc agaaacccctc
801 gacgacctgt tgcccaggc cttegccgtg gcccgcgagg ccgcttggcg
851 ggtgctggac cagcggccgt tcgacgtgca ggtgatgggt gcggccgccc
901 tgcacctggg caacgttgcc gagatgaaga ccggtgaagg caagacccctg
951 acctgtgtgt tgcccgtta cctcaatgcg ctggccggca acggcgtgca
1001 catcgtcacc gtcaacgact acctggctaa acgcgacagt gagtggatgg
1051 gccgcgtgca ccgcttcctc gggttcagg tcgggggtgat tttegccacc
1101 atgacacccg atgaacgcg ggtggcctat aacgccgaca tcacctacgg

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FIG. 6A

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1151 caccaataac gagtttgggt tcgaactacct gcgcgacaac atggcgcact
1201 cactggatga tctggtgcag cgcgggcacc attacgccat tgtcgacgag
1251 gtcgattcca tctgatcga cgaggccgcg acccgcgtga tcattctcgg
1301 tccgcgcgac ggcctccaac tggtaaccg agttcgccgg ttggcgccgc
1351 tgatggaaaa ggacgtccac tacgaggtcg atctacgcaa acgcaccgtc
1401 ggcgtgcacg agaagggtgt ggaattcgtc gaagaccagc tcggcatcga
1451 caacctgtac gagggcgcca actcgccgtt ggtcagctat ctcaacaacg
1501 ctctgaaggc caagagctg ttacgcgcg acaaggacta catcgtcgc
1551 gatggtgagg tgctcatcgt cgacgagttc accggccggg tgcctgatcgg
1601 ccgcgcctac aacgagggca tcgaccggc catcgaggcc agggagcacg
1651 tcgagatcaa ggccgagAAC cagacgttg ccaccatcac gctgcagaac
1701 tacttccggc ttacgacaa gctcgccggc atgaccggca ccgccacgac
1751 ggaggcgccc gagctgcacg agatctacaa gctgggctg gtcagcatcc
1801 cgaccaacat gccgatgat cgtgaagacc agtccgacct gatctacaag
1851 accgaggagg ccaagtacat cgcggtggtc gacgacgtcg ccgagcgcta
1901 cgcgaaggga cagccggtgc tgatcggcac caccagcgtg ggcgcctcgg
1951 agtatctgtc gcggcagttc accaagcggc gcattccgca caatgtgtc
2001 aacgcccaagt accacgagca agaggcgacc atcatcgcgg tggcggggccg
2051 ccgcggcggc gtaccgctcg ccaccaacat ggccggctgc ggcaccgaca
2101 ttgtgctggg cggcaacgtc gactttctca ccgatacagc gctgcgcgaa
2151 cggcctggat ccggtggaga cgcccagga gtacgaggcg gcctggcact
2201 ccgaactgcc catcgtaaa gaggaagcca gcaaggaggc caagggaagta
2251 atcgaggccg gcggtgtac gtgctgggca ccgagcggcc acgagtcgcg

FIG. 6B

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2301 gcggatcgac aaccagttgc gtggccggtc cgcccgccag gggaccccg
2351 ggagtcgcgc ttctatttgt cgctgggtga cgagctgatg cgccgcttca
2401 atggcgcgcc cttggagacc ttgttgacca ggtgaacct gcccgacgac
2451 gtgccgatcg aagccaagat ggtaccccg gccatcaaga gcgccccagac
2501 ccaggtcgag cagcagaact ttgaggtccg caagaacgtc ctcaaatacg
2551 acgaggtgat gaaccagcag cgcaaggta tctacgccga gcgcccggcg
2601 atcctegaag gcgaaaacct caaggaccag gcgtggaca tggtcgcga
2651 tgtcatcacc gcttaegtcg acggcgcgac cgcggaaggc tatgccgaag
2701 attgggatct ggacgcgttg tggacggcac tcaaaacct ctatccggag
2751 gggatcaccc cgaactcgt gacccgcaag gaccacgaat tcgagcgca
2801 cgatctcacc cgcgaggagt tctggaggc actactcaag gacgccgaac
2851 gtgcctatgc cgcacgggaa gccgaactcg aggaatcgc cggcgagggt
2901 gcgatgcgcc agctggaac caactgtctg ctcaacgtca tagaccgtaa
2951 gtggcgtgaa cactctacg agatggacta cctcaaggag ggtatcgggc
3001 tgcgcgcgat ggcgcacggc gatccgttg tgcagtacca gcgtgagggc
3051 tacgacatgt tcatggccat gctcgacggc atgaagagg aatcggtcgg
3101 ctctctgttc aacgtcaccc tggaggcgt cccgcgcccg ccggttgccc
3151 cggctgccga accgcagag cttgccgaat tcgcgcgcgc gcccgacgac
3201 gcgggcagca acgcagcgcg gtcgatggtg gcgcgcgcga aagagctcca
3251 agtgcattac gcgccaaggg tgtgccagc ggtcgcccc cttgacct
3301 ttccggtccc gcggaggatg gctcggctca ggtgcagcg aacggcggtg
3351 gagccccaaa gacgccggcc ggagtgcgg ccggtgctag ccggcgcgag
3401 cggcgcgaac gcgccgcgc acaaggccgc ggcgccaaag cgccgaatc

FIG. 6C

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3451 ggtcaagaag cgttagcgcg taggttgacg atgggtgtat cggtttctca
3501 gttcccagaa gtcacttccc ggcacacccc ggccccggcg cgcattgcaca
3551 ttctcgttga cggcgggcaa ggggttcgct aatctcacc gttcgtcgac
3601 ctctcgtcgc gtcggttctg ctggtagcgg ggttcggcgc ttctctggcg
3651 ttctctgact cgacaatcgt caacatcgcg ttcccgata tccagcgttc
3701 ctctccgtcc tacgacatcg ggagcctgc ctggattctg aacggctata
3751 acatgctctt cgcgccttc atggttgagg ccggcagggtt ggccgatttg
3801 ctgggcccga gacgacattc ctgtccggtg tgcgtgtgtt caccattgcg
3851 tccgggctgt gcgcgctgc cggcagtgtc gagcagttgg tggcgttccg
3901 ggtgctgcag ggcctcgggg ctgcgatact cgtgcctcgt tcgctcgac
3951 tggtcgttga gggcttcgac cgggcgcggc cgcgcacgct atcggcctgt
4001 ggggtgcggc ggcagcgatc cactagtctt agagcggcgc accgc

FIG. 6D

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1 tcaaacacca gaccagaagg aggcacaacg atcacggacg gtgccgttcg
51 tcgagcggga gcctggggcg gatcgttcgc accaagaac acccggcca
101 cgccgatgtc ggtcgatgac gcgctctacc agatggagct ggttgacac
151 gactttctct tgttttaega caaggacacc gaacggccgt cggtggtcta
201 ccgccggcac gcctacgaet acgcttgat ccgtctggcg tcatcggcgg
251 cgcgccgcgc gtcgtcacct accatgggag tcgccttacc taaagactcc
301 tacacatgcg gggacatagc tgtgtgtcg aagttgctgc gccttggcga
351 aggtcgcatg gtcaagcgcc tcaagaaggt ggcgactat gtcggcaactt
401 tgtccgacga tgtcgagaaa ctcaaccgacg ccgagctgag ggcgaaaacc
451 gacgagttca agcaggctgg ccgaccagaa aaaccagaa accctcgacg
501 acctgtttgc cgaggccttc accgtgcccc gcgagaccgc cctgccgggt
551 gctggaccac cgaccgttcg acgtgcaggt gatgggtacg accgccctgc
601 acctgggcga cgttgccgag atgtagaccg gtgaaggcaa gacctgacc
651 tgtgttttac ccgcttacct caatgccctg gccgccaacg gcgtgcacgt
701 agttaccgtc aacgactacc tggetaaacg cgacagtga tggatgggcc
751 gcgtgcaccg ctccctcggg ctteaggtcg gggatgattt ggccaccatg
801 acacccgatg aacgcgggt ggcctataac gccgacatca cctacggcac
851 caataacgag tttaggttcg actacctgcg cgacaacatg gcgcactcac
901 tggatgatct ggtgcagcgc gggaccatt acgccattgt cgacgaaggt
951 cgattccatc ctgatcgacg agggcggggc cccccccca tctccgcccg

FIG. 7A

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1001 gggcgccgc ctccaactgg ttcaccgagt tcgcccggtt ggcgtgccgc
1051 ggctggtttt ggacgtccac tacgaggtcg atctacgcaa acgcaccgtc
1101 ggcgtgcaag agaagggtgt ggaattcgte gaagaccagc tcggcatcga
1151 caacctgtac gagaccgcca actcgccggtt ggtcagctat ctcaacaacg
1201 ctctgaagge caaagagctg ttcagccgcg acaaggacta catcgtccgc
1251 gatggtgagg tgetcatcgt cgacgagttc accggccggg tgetgatcgg
1301 ccgcgcgtac aacgagggca tgcaccagge catcgaggcc aaggagcacg
1351 tcgagatcaa ggccgagaaac cagacgttgg ccaccatcac gctgcagaac
1401 tacttcggc tctaggagaa gctcgccggg atg

FIG. 7B

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1  tggettgtt caaactagt aacaataaat taagtttaaa gcacttgtgt
51  ttttgcacaa gtttttttat actccaaaag caattatgo ctatttcata
101 gttcgataat gtaatttgtt gaatgaaca tagtgactat gctaattgta
151 atggatgtat atatttgaat gttaagttaa taatagtatg tcagtcattt
201 gtatagtccg agtcgaaaat cgtaaaatat ttataatata atttatagg
251 aagtataatt gcgtattgag aatatattta ttagtataa acttgttgac
301 aacagaatgt gaatgaagta tgtcataaat atatttatat tgattctaca
351 aatgagttaa taagtataat tttctaacta taaatgataa gatataattgt
401 tgtaggccaa acagtttttt agctaagga gcgaacgaaa tgggattttt
451 atcaaaaaatt cttgatggca ataataaaga aattaaacag ttaggtaaac
501 ttgctgataa agtaatcgct ttagaagaaa aaacggcaat ttttaactgat
551 gaagaaatlc gtaataaaac gaacaattc caaacagaat tagctgacat
601 tgataatgtc aaaaagcaaa atgattattt acataaaaatt ttaccagaag
651 catatgcaet tgttagagaa ggctctaaac gtgtattcaa tatgaaccca
701 tataaagtc aaattatggg tggatattga attcataaag gtgatategc
751 tgagatgaga acaggtgaag gtaaaacatt aacagcgaca atgccaacat
801 acttaaatgc attagctggt agagggttc acgttattac agtcaatgaa
851 tacttatcaa gtgttcaag tgaggaatg gctgagttat ataacttctt
901 aggtttgact gtcggattaa acttaaacag taagacgaca gaggaanaac
951 gtgaagcata cgcacaagac attacttaca gtactaataa tgagctaggt
1001 tttgattact tacgagataa catggtgaat tattctgaag atagggtaat
1051 gcgtccatta cattttgcaa tcattgatga ggtggactca attttaatcg

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FIG. 8A

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1101 acgaggcacg tacgccatta attatttctg gtgaagctga aaagtcacag
1151 tcactttata cacaagcaaa tgtttttgcg aaatgttaa acaggagcga
1201 tgattataaa tacgatgaaa aaacgaagc tgtacattta acagaacaag
1251 gtgcggtata agctgaacgt atgttcaaa ttgaaaactt atatgatga
1301 caaatgttg atgttattag tcatatcaac acagctttac gtgcgcacgt
1351 tacattacaa cgtgacgtag actatatggt tgttgatggc gaagtattaa
1401 ttgtcgatca atttacagga cgtacaatgc caggccgtcg ttctcggaa
1451 ggtttacacc aagctattga agcgaaggaa ggcgttcaaa ttcaaaatga
1501 atctaanaact atggcgctca ttacattcca aaactatttc agaagtaca
1551 ataaacttgc gggatatgaca ggtacagcta aaactgaaga agaagaattt
1601 agaatattt ataacatgac agtaactcaa attccgacaa ataaccctgt
1651 gcaacgtaac gataagtctg atttaattta cattagccaa aaaggtaaat
1701 ttgatgcagt agtagaagat gttgttgaaa aacacaaggc agggcaacca
1751 gtgctattag gtaactgttc agttgagact tctgaatata ttccaattt
1801 acttaaaaaa cgtggtatcc gtcattgatgt gttaaatgag aaaaatcatg
1851 aacgtgaagc tgaatttgtt gcaggcgctg gacaaaaagg tgccgttact
1901 attgccacta acatggctgg tcggggtaca gatatacaat taggtgaagg
1951 cgtagaggaa ttaggcggtt tagcagtaat aggtacagag cgacatgaat
2001 ctgctcgat tgatgaccag ttacgtggtc gttctggacg tcaagggtgat
2051 aaaggggata gtcgcttcta ttatcatta caagatgaat taatgattcg
2101 ttttggttct gaacgtttac agaaatgat gagccgacta ggttagatg
2151 actctacacc aattgaatca aaatgggtat caagagctgt tgaatcagca

FIG. 8B

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2201 caaaacgtg tagaaggtaa taacttegac ggcgtaaac gtatcttaga
2251 atacgatgaa gtattacgta aacaacgtga aattatctat aacgaagaa
2301 atagtattat tgatgaagaa gacagctctc aagttgtaga tgcaatgcta
2351 cgttcaacgt tacaacgtag tatcaattac tatattaata cgcagatga
2401 cgagcctgaa tatcaacccat tcatgacta cattaatgac atctcttac
2451 aagaaggtag cattacagag gatgatatca aaggtaaaga tgetgaagat
2501 attttcgaa tcgtttggc taagattgaa gcagcatatc aaagtc meta
2551 agatatctta gaagaacaaa tgaatgagtt tgagcgtagt attttactc
2601 gttctattga tagccattgg actgataata tcgacacaaat ggatcaatta
2651 cgtcaaggta tteacttacg ttcttatgca caacaaatc cattacgtga
2701 ctatcaaaat gaaggtcatg aattatttga tatcatgatg caaatattg
2751 aagaagatac ttgtaaattc attttaaat ctgtagtaca agttgaagat
2801 aatattgaac gtgaaaaaac aacagagttt ggtgaagcga agcacgttcc
2851 agctgaagat ggtaaagaaa aagtgaacc gaaaccaatc gttaaaggcg
2901 atcaagttgg tcgtaacgat gattgtccat gtggtagtgg taaaaaatc
2951 aaaaattgcc atggaataa atgatataa aataactcct tccaat meta
3001 caccatatagt ttgtgttatg ggaggagctc ttttatatta caagcg meta
3051 atacttt meta aatgtgaag aagttgttaa acgttgttat gtacttagtt
3101 ttaaaaaatc ggtttaggca tatg

FIG. 8C

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1 cttgaacgtt acttcaactaa tgtgcgcaat gtgaatgcac atgtaaaagt
 51 gaaaacttat gcaaatctta gcaaaaatc gaagttacaa ttccgcttaa
 101 tgacgtgaca cttegtgcag aagaagaaa cgatgattta tgcgtgaatt
 151 gacaagatca ctaacaattt agaatgtcaa gttegtaaat acaaaacacg
 201 tgtcaatcgt aagaaacgta aagaagcga acatgaacca ttcccagcaa
 251 ctccggaaac tccgcggaa acagctgttg atcatgataa agatgatgaa
 301 attgaatca tccgttctaa acaattcagc ttgaaaaccaa tggattctga
 351 agaagcggta ttacaaatgg atttaactgg tactgatttc ttcatcttca
 401 atgaccgtga aactgatggt acaagcattg ttaccgccc taaagacgga
 451 aatatgtgtt tgattgaac tgttgaaaa ctaatatgtg atatttgaaa
 501 gggctcttgc tgcattttct gctgcaagag ttctcttttt tgagaaagcc
 551 cttattaaga ttgtattaat aaaaatacaa ttgattgatt tacacggggt
 601 gtccatgtca aaataagagg gatgtattaa gttcataatt gtaatgtgag
 651 ctccgatgag tgagcggcat atgattatga tatccatgtg gcacatgatg
 701 ttaacaaaaa gagaatgaaa ctgtgagaag tacatcttga taaacacaac
 751 taggcagttt attaaaaaat aatgaacagt atccatgag tttttaagta
 801 taaattaagc catataaatg gtaagataaa ttgttgtaag ccaaacagtt
 851 tttataccaa aggagcgaac agaattgggtt ttttaacaaa aattgttgac
 901 ggcaataaga gagaatcaa acgcctaagt aagcaagctg acaagtaat
 951 ctcattagaa gaagaatgt caattcttac tgatgaagaa attagaata
 1001 aacaaaagc attccaagaa agattgcaag cagaagaaca tgaagcaaa
 1051 caagataaaa ttttagaaga aatattacct gaagcatttg cgcttgccg
 1101 tgaaggagct aaacgtgtat ttaatatgac acctatcca gtccaatca
 1151 tgggtggtat cgccattcat aatggtgaca tttcagaat gagaacaggt

FIG. 9A

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1201 gaaggtaaaa cattaactgc aacgatgccg acttatttaa acgccttagc
1251 agcacgtggt gtgcattgta ttacagtcaa tgaatacttg gcaagttctc
1301 aaagagaaga aatggccgag ttatataatt tccttggttt atcagtcgga
1351 ttgaacttga acagcttate aacagaacaa aagcgtgaag cttataatgc
1401 agatatattac tatagtacaa ataatgaatt aggcctcgac tatttacgcg
1451 ataacatggt gaattattca gaagaacgtg ttatgcgtcc gcttcatttc
1501 gctatcattg atgaggtcga ctctatttta atcgatgaag cgcgtacacc
1551 attgattatt tcaggggaag ctgaaaaac aacatctctt tatacacaa
1601 caaatgtttt cgctaaaatg ttaaaagcag aagatgatta taattatgat
1651 gaaaaaacaa aatcagtaaa attaacagat caagggtctg ataaagctga
1701 acgtatgttc aagttagata acttatatga ttgaaaaac gttgatatta
1751 tcacgcatat caatacagca ttaegtgcta actatacatt gcaacgcgat
1801 gtagattaca tggttgtaga tggagaagta ttgattgtcg accaatttac
1851 aggtcgaaac atgccaggtc gtcgattctc tgaaggactt caccaagcga
1901 ttgaggctaa agaaggggtt caaattcaaa atgaatctaa aacaatggct
1951 tctatcacat tccaaaacta ctccgctatg tataataaat tagccggtat
2001 gacaggtaac gctaaaacag aggaagaaga attccgtaac atttataata
2051 tgacagttac acaaatcca acgaaccgtc ctgttcaacg tgaagataga
2101 cctgacttga ttttcatcag ccaaaaaggc aagttcgatg ctgttggtga
2151 agatgttgtt gaaaaacata aaaaaggcca accaatctt ttaggtactg
2201 tagcggltga aacaagtgaa tacatttcac aactattgaa aaaaacgcgt
2251 gtgcgtcatg atgtcttaaa cgctaaaaac catgaacgcg aagctgaat
2301 cgtatctaca gcaggtaaaa aaggtgcagt cacaatcgca acaaacatgg
2351 ctggtcgtgg taccgatatt aattaggcg aaggtgttga agaattaggc
2401 ggccttgctg ttattgtac agaagctcat gaatcacgcc gtatcgatga

FIG. 9B

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2451 tcagttgct ggtcgttctg gacgacaagg tgaccgcgga gaaagccgtt
2501 tctatttate attacaagat gagttgatgg tacgtttcgg ttctgaacgt
2551 ctgcacaaaa tgatgggccc attaggtatg gatgactcta caccgatga
2601 atcaaaaaatg gtatctcgag ctgttgaate tgcacaaaaa cgtgttgaag
2651 gtaacaactt cgatgcacgt aaacgtatct togaatacga tgaagtttta
2701 cgtaaacacac gtgaataatcatttatggtaa cgttaataata ttatcgattc
2751 agaatacaagt tetgaattag tcattacaat gatcgcctc acattagatc
2801 gtgcaatcag ttatttatgta aatgaagaat tggagaagaat tgactatgag
2851 ccgtttatta attttggtga agatgttttc ttdecaggaag gtgaagtcaa
2901 agaagatgaa atcaaaaggta aaggtaaaga tcgtgaggat attttcgata
2951 cagtatggc taaaattgaa aaagcttatg aagcacaaaa agccaatata
3001 cccgaccaat tcaatgaatt cgaacgtatg attttattac gttctattga
3051 tggagatgg acagaccata tcgatacaat ggtcaaatla cgtcaaggta
3101 tccatttacg ttcatacggt caacaaaacc cacttcgca ctatcaaat
3151 gaagggcacc aactatttga tacaatgatg gtcaatatgg aagaagacgt
3201 cagcaaatat atcttgaat caattatcac agtagatgat gatattgaac
3251 gtgataaagc aaaagaatat caaggacaac atgtatcagc tgaagatgga
3301 aaagaaaaag taaaccgca accagttgtt aaagataatc acatcggaag
3351 aatgatcct tgtccatgag gcagcggtaa aaagtataaa aattgctgag
3401 gtaaatagta agttgtatta ggaccactgt taatatagctt taagagagat
3451 gctcaattga aattgggtta tctttctaag ggctgtcagc ggtctttttt
3501 caatccaaca aaaatatgga tatatgctaa aataatagag taatcttgaa
3551 aattaaactg gaattggaga gatatgaaa tgggaattat

FIG. 9C

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```

1  cagtcaatgt cgctcttctgt gaccgagcca atggacggaa aggtgccccg ctcccagatc
61  atgaacctcc tagtgtacgc ctataagaag ggccttaaga cggggctcta ctactgcaag
121 atccgcaagg ccaccaaca cggcgtcttc acgggcggcg accctgtgtg ctctgggtgc
181 cacctgtagc gacgcgcgc ggcgcgcgatg gccgagggcgg cggacgcggc gacctcaacg
241 cgtaaatata aatactttta cgagaccgag tgccccgacc tagatcaatt gcggtcgtc
301 agcgtcgcaa accgctggct ggagaccgag ttccccctag cggacgcgc caaggacgtg
361 gcgcggctca gcggcgccga gctggagtct taecgcttcc tgctgcgtt cctctcgcc
421 gccgatgacc tcgtgaacgt caacctcggg gacctgtccg agctgttcc ccaaaaagac
481 atcctgcatt actatatacga gcaggagtcc atcgaggtgg tgcaactcgc ggtgtacagc
541 gccatcacgc tgctgtctct tagaacgac gcggtggcgc gcgcgggcta cgtagagggc
601 gccctcggcg acccggcgggt ccggcgcaag gtggactggc tcgagcggcg cgtggccgcg
661 gcagagtcgg tggccgaaaa gtacgtgctc atgattctaa tcgagggcat ttttttctcc
721 tcctcgtttg cggcgattgc ctacctgcgc acccaaaccc ttttcgtcgt gacgtgccaa
781 accaacgacc tcatcagccg cgacgaagcc gtgcacacgg ccgcgtcgtg ctgcatcttc
841 gacaaactacc tcggcgggga gcggccgcgc ccggcccgcg tctacgagct gttccgcgaa
901 gcgtggaaat tgagcgcgag tttatttggg tgccgcgcgc gcggcagtca tatacttgac
961 gtggaggcta tttctgcgtc cgtcgagtac agcgcggacc gcctgctcgc tgcctaccag
1021 ctgcctcctc tgtttggcac ccgcctcctt gggaccgatt ttcccttggc cctgatgact
1081 gccgagaagc acacgaactt ctttgagcgc cgcagaccca actacacagg caccgtaatc
1141 aacgacctgt agggcaccct cgtgccttg ccagagcgcc ccgccttccc tcctccttct
1201 cccccccacg ccgcgaataa aaatgttcc atgtcaacga aa

```

FIG. 10

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1 tcgagccgc cgaaccgc cgcgtctgtt gaaatggcca gccgccagc cgcacctct
 61 cccgtcgaag cgcgggcccc ggttgggga caggaggccg gcggccccag cgcagccacc
 121 cagggggagg ccgccgggc cctctcgcc cagggccacc acgtgtactg ccagcgagtc
 181 aatggcgtag tgggtcttcc cgacaagacg ccgggtccg cgtcctaccg catcagcgat
 241 agcaactttg tccaatgtgg ttccaactgc accatgatca tcgacggaga cgtggtgcgc
 301 gggcgcccc aggacccggg gggcgggca tccccgctc ccttcgttc ggtgacaaac
 361 atcggagccg gcagcgacgg cgggaccgc gtcgtggcat tcgggggaac cccacgtcgc
 421 tcggcgggga cgtctaccgg taccagacg gccgacgtcc ccaccgagcc ccttgggggc
 481 cccctctc ctcgccctt caccctgggt ggcggctgtt gttcctgtcg cgacacacgg
 541 cgcgcctc cgtatttcg gggggagggg gatccagtcg gcccgcgga gttcgtctcg
 601 gacgacgggt cgtccgattc cgactcggat gactcggagg aacgggactc ggagacgctg
 661 tcacacgctt cctcggacgt gtccggcggg gccacgtacg acgacgcct tgaactccgat
 721 tcgtcatcgg atgactccct gcagatagat ggccccgtgt gtcgcccggt gagcaatgac
 781 accgcgcccc tggatgtttg ccccgggacc cccggccccg gcgcgcgacgc cgttggtecc
 841 tcagcggtag acccacacgc gccgacgcca gaggccggcg ctggtcttc gcccgatccc
 901 gccgtggccc ggaagacgc ggaagggtt tcggaccccc ggccacgtct gggaacgggc
 961 acggcctacc ccgtccctt ggaactcacg cccgagaaac cgagggccgt ggcgcgcttt
 1021 ctggggagatg ccgtgaaccg cgaaccccg ctcattgtgg agtactttg ccggtgcgcc
 1081 cgcgaggaaa ccaagcgtgt cccccccagg acattcgga cccccctcg cctcacggag
 1141 gacgactttg ggcttctcaa ctacgcgtc gtggagatgc agcgctgtg tctggacgtt
 1201 cctccggtcc cgcggaacgc atacatgccc tattatctca gggagtatgt gacgcggctg
 1261 gtaacgggt tcaagccgt ggtgagccgg tcgcctcgcc ttaccgcct cctgggggtt
 1321 ctggtgcacc tcgggatccg gaccggggag gcctcctttg aggagtggct gcgatccaa

FIG. 11A

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1381 gaagtggccc tggatttttg cctgacggaa aggettegag agcagaagc ccagctggctg
 1441 atcctggccc aggtcttgga ccattacgac tgtctgatcc acagcacacc gcacacgctg
 1501 gtcgagcggg ggtgcaatc ggcctgaag tatgaggagt ttacctaata gcgttttggc
 1561 gggcaactaca tggagtccgt ctccagatg tacacccgca tcgccggctt ttggccctgc
 1621 cgggccacgc gcggcatgcg ccacatcgcc ctggggcgag aggggtcgctg gtgggaaatg
 1681 ttcaagttct ttttccaccg cctctacgac caccagatcg taecgtcgac ccccgccatg
 1741 ctgaacctgg ggaaccgcaa ctactacac tccagctgct acctggtaaa cccccaggcc
 1801 accacaaca aggcgacct gcggccatc accagcaacg tcagtgccat cctgcgccgc
 1861 aacgggggca tcgggtatg cgtgcaggcg tttaacgact ccggccccgg gaccgccagc
 1921 gtcatgcccc cctcaaggc cctgactcg ctggtggcgg cgcacaacaa agagagcgcg
 1981 cgtccgaccg gcgcgtgctg gtacctggag ccgtggcaca ccgacgtgag ggcgtgctc
 2041 cggatgaagg ggtcctcgc cggcgaagag gccacgctt gcgacaatat ctccagcgcc
 2101 ctctggatgc cagacctgtt ttccaagcgc ctgattcgcc acctggacgg cgagaagaac
 2161 gtacacatgga cctgttcga ccgggacacc agcatgtcgc tcgccgactt tcacggggag
 2221 gatttcgaga agctctacca gcacctcgag gtcatgggggt tcggcgagca gatacccatc
 2281 caggagctgg cctatggcat tgtgcgcagt gcggccacga ccgggagccc ctctgctcatg
 2341 ttcaagacgc cggtgaaaccg ccactacatc tacgacaccc agggggcgcc catcgccggc
 2401 tccaacctct gcaccgagat cgtccatccg gcctccaagc gatccagtgg ggtctgcaac
 2461 ctgggaagcg tgaatctggc ccgatgcgtc tccaggcaga cgtttgactt tggcgggctc
 2521 cgcgacgccc tgcaggcgtg cgtgctgatg gtgaacatca tgatcgacag caccgtacaa
 2581 cccacgcccc agtgacccc cggcaacgac aacctgcggt ccatgggaat cggcatgcag
 2641 ggcctgcaca cggcctgcct gaagctgggg ctggatctgg agtctgccga attcaggac
 2701 ctgaacaaac acatcgccga ggtgatgctg ctgtcggcga tgaagaccag caacgcgctg

FIG. 11B

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2761 tgcgttcgcg gggcccgtcc cttaaccac tttaagegca gcatgtatcg cgccggccgc
2821 tttaactggg agcgtttcc ggacgcccgg ccgcggtacg agggcgagtg ggagatgcta
2881 cgccagagca tgatgaaca cggcctgcgc aacagccagt ttgtcgcgct gatgccacc
2941 gccgcctcgg cgcagatctc ggacgtcagc gagggtttg ccccccgtt caccaccctg
3001 ttcagcaagg tgaccggga cggcgagacg ctgcgcccc acaegctcct gctaaaggaa
3061 ctggaacgca cgtttagcgg gaagcgcctc ctggaggtga tggacagtct cgacgccaag
3121 cagtggtcgg tgccgcaggc gctcccgtgc ctggagccca cccacccct ccggcgattc
3181 aagaccgcgt ttgactacga ccagaagtgc ctgacgacc tgtgtgcgga ccgcgcccc
3241 tacgtcgacc atagccaatc catgaccctg tatgtcacgg agaaggcgga cgggaccctc
3301 ccagccctcca ccctggtcgg ccttctggtc cagcgcgact agcgcggact aaaaacaggg
3361 atgtactact gcaaggttcg caaggcgacc aacagcgggg tctttggcgg cgacgacaac
3421 attgtctgca tgagctgcgc gctgtgaccg acaaaccccc tccgcgccag gcccgccgcc
3481 actgtcgtcg ccgtcccaag ctctcccctg ctgccatg

FIG. 11C

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1  gtgtgttttgg  cgtgtgtctc  tgaatggcg  gaacccaca  tgcaaatggg  attcatggac
61  acgttacacc  cccctgactc  aggagatagg  catatcctcc  ttagattgac  tcagcacacg
121  atcgaccccc  acccttgtgt  gccggggata  aagccaacg  cgcgggtct  gggttaccac
181  aacagggtggg  tgcttcgggg  acttgacggt  cgcaactctc  ctgagagccc  tcacgtcttc
241  gcccacccgat  tcctgttgcg  ttccctgtcg  ccggtgetgt  cctgtcgaca  gattgttggc
301  gactgccccg  gtgattcgtc  ggccggtgcg  tccttteggt  cgtaccgccc  accccgcctc
361  ccaaggggccc  gccgtgttt  ccgttcateg  cgtecgagcc  accgtcacct  tggttccaat
421  ggccaaccgc  cctgccgat  ccgccctgc  cggagcgcg  tctccgtccg  aacgacagga
481  accccgggag  ccgagggtcg  cccccctgg  cggcgaccac  gtgttttga  ggaagtcag
541  cggcgtgatg  gtgtttcca  gcgatcccc  cggccccgcg  gcctaccgca  ttgcgcacg
601  cagctttgtt  caatgcggt  ccaactgcag  tatgataatc  gacggagacg  tggcgcgcg
661  tcatltgcgt  gacctcgagg  gcgtacgtc  caccggcgcc  ttctgcgca  tctcaaacgt
721  cgcagccggc  ggggatggcc  gaaccgcct  cgtggcgctc  ggcggaacct  cgggcccgtc
781  cgcgaactaca  tccgtgggga  ccagacgtc  cggggagtcc  ctccacggga  acccaaggac
841  ccccgaaacc  caaggacccc  aggtgtccc  cccgccccct  ctccccct  ttccatgggg
901  ccaegagtgc  tgcgccctc  gcgatgccag  gggcggcgcc  gagaaggacg  tcggggccgc
961  ggagtcatgg  tcagacggcc  cgtcgtccga  ctccgaaacg  gaggactcgg  actcctcgga
1021  cgaggatacg  ggctcgggtt  cggagacgt  gtctcgatcc  tcttcgatct  gggccgcagg
1081  ggcgactgac  gacgatgaca  gcgactccga  ctgcggtcg  gacgactccg  tgcagccccg
1141  cgttgtcgtt  cgtcgcagat  ggagcgacgg  ccctgcccc  gtggcctttc  ccaagccccg
1201  gcgccccggc  gactccccg  gaaacccccg  cctgggcgcc  ggcaccgggc  cgggctccgc
1261  gacggacccc  cgcgcgtcgg  ccgactccga  ttccgcggcc  caccgcgcg  cccccaggc
1321  ggacgtggcg  ccggttcttg  acagccagcc  cactgtggga  acggaccccc  gctaccacgt

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FIG. 12A

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1381 cccctagaa ctacgcccc agaagcgga ggcggtggcg cgtttcttgg gggacgccgt
 1441 cgaccgcgag cccgcgctca tgetggagta cttctgtcgg tgcgcccgcg aggagagcaa
 1501 gcgctgccc ccacgaacct tcggcagcgc ccccgcctc acggaggacg actttgggct
 1561 cctgaactac gcgctcgctg agatgcgacg cctgtgcctg gacctcccc cgtccccccc
 1621 caacgcatac acgcccatac atctgagga gatatgcgacg cggttggtta acgggttcaa
 1681 accctgggtg cggcggtcgg cccgcctgta tcgcatacctg gggattcttg ttacactgcg
 1741 cctccgtacc cgggaggcct cctttgagga atggatgcgc tccaaggagg tggacctgga
 1801 cttcgggctg acggaaaggc ttgcggaaca cgaggcccag ctaatgatec tggcccaggc
 1861 cctgaacccc tacgactgtc tgatccacag cccccgaac acgctcgteg agcgggggct
 1921 gcagtcggcg ctgaagtacg aagatttta cctcaagcgc ttcggcgggc actacatgga
 1981 gtccgtcttc cagatgtaca cccgcctgc cgggttcctg gcgtgccggg cgaccgcggg
 2041 catgcgccac atcgccctgg ggcgacaggg gtctgtgttg gaaatgttca agttctttt
 2101 ccaccgcctc tacgaccacc agatcgtgcc gtccaccccc gccatgtga acctcggaac
 2161 ccgcaactac tacacgtcca gctgatacct ggtaaacccc caggccacca ctaaccaggc
 2221 caccctccgg gccatcacccg gcaacgtgag cgccatcctc gcccgcaacg ggggcatacgg
 2281 gctgtgcatg caggcggtta acgacgccag ccccgccacc gccagcatca tgccggccct
 2341 gaaggtcctg gactccctgg tggcggcgca caacaacag agcacgcgcc ccaccggggc
 2401 gtgcgtgtac ctggaaacct ggcaacgca cgttcgggc gtgtcagaa tgaagggcgt
 2461 cctcgccggc gaggaggccc agcgtgcga caacatcttc agcgccctct ggtgccgga
 2521 cctgttcttc aagcgctga tccgccacct cgacggcgag aaaaacgtca cctggtcctc
 2581 gtctgacccg gacaccagca tgctcgctgc cgactttcac ggcgaggagt tcgagaagct
 2641 gtacgagcac ctgaggcca tggggttcgg cgaacgata cccatccagg acctggcgta
 2701 cgccatcgtg cgcagcgcg ccaccaccgg aagccccttc atcatgttta aggacgcggt

FIG. 12B

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2761 aaacagccac tacatctacg aacgcaagg ggcggccatt gccggctcca accctgcac
 2821 ggagatcgtc caccgtcct ccaacgctc cagcggggtc tgcaacctgg gcagcgtgaa
 2881 tctggcccga tgcgtctccc ggcggacgtt cgattttggc atgctccgcg acgccgtgca
 2941 ggctgcgtg ctaatggta atatcatgat agacagcacg ctgcagccga cgcgccagtg
 3001 cgcgcgcggc cagcaaac tgcggtccat gggcattggc atgcagggcc tgcaacaggc
 3061 gtgcctgaag atgggctgg atctggagtc ggcgagttc cgggacctga acacacacat
 3121 cgcgaggtg atgctgctcg cggccatgaa gaccagtaac gcgtgtgcg ttgcgggggc
 3181 gcgtcccttc agccactta agcgagcat gtaccgggcc ggcgcttctc actgggagcg
 3241 cttttcgaa gccagccgc ggtacgagg gtagtggag atgctacgcc agagcatgat
 3301 gaaacacggc ctgcgaaca gccagtcat cgcgtcatg cccaccgccg cctcggccca
 3361 gatctcggac gtcagccagg gctttgccc cctgttcacc aacctgtca gcaagtgac
 3421 caggacggc gagacgtgc gcccacac gctcttgctg aaggaactcg agcgacgtt
 3481 cggcgggaag cggtcctgg acgcgatgga cgggctcgag gccaaagcagt ggtctgtggc
 3541 ccaggccctg ccttgccctg accccgccca cccctccgg cggttcaaga cggccttcga
 3601 ctacgaccag gaactgtga tcgacctgtg tgcagaccgc gccccctatg ttgatcacag
 3661 ccaatccatg actctgtatg tcacagagaa ggcggacggg acgctccccg cctccaccct
 3721 ggtccgccct ctcgtccacg catataagcg cggcctgaag acggggatgt actactgcaa
 3781 ggttcgcaag gcgaccaaca gcggggtgtt cgcggcgac gacaacatcg tctgcacaag
 3841 ctgcgcgtg taagcaacag cgctccgata ggggtcaggc gtcgctctcg gtcccgcata
 3901 tcgccatgga tcccgccgtc tccccgcga gcaccgaccc cctagatacc cagcgctcgg
 3961 gggccggggc ggcccagatt ccggtgtgce ccaaccccca gcggtacttc tacacctccc
 4021 agtggcccga catcaaccac cttegtccc tcagcatcct gaaccgctgg ctggagaccg
 4081 agctcgtgtt cgtcggggac gaggaggacg tctccaagct ctccgagggc gagctcggct

FIG. 12C

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4141 ttaccgctt tctgtttgcc ttctgtcgg ccgaggacga cctggtgacg gaaacctgg
 4201 gcggcctctc cgccctcttc gaacagaagg acattcttca ctactacgtg gaggaggaat
 4261 gcatcgaggt cgtccactcc cgcgtctaca acatcatcca gctggtgctc ttteacaaca
 4321 acgaccaggc gcgcgcgcc tatgtggccc gcaccatcaa cccccggcc attcegtca
 4381 aggtggactg gctggaggcg cgggtgcggg aatgcgactc gatcccgagg aagttcatcc
 4441 tcatgatact catcgaggcg gtcttttttg ccgcctcggt cgcgcgcactc gcgtacctgc
 4501 gacccaacaa cctcctgcgg gtcacctgcc agtcgaacga cctcatcagc cgccacgagg
 4561 ccgtgcatac gacagcctcg tgctacatct acaacaacta cctcgggggc cagcccaagc
 4621 ccgaggcgcc gcgcgtgtac cgctgtttc gggaggcgggt ggatatcgag atcgggttca
 4681 tccgatccca ggcgcgcgac gacagctcta tctgagtc cggggccctg gcggccatcg
 4741 agaactacgt gcgattcagc gcggatcgcc tgcctggcct gatccatatg cagccccctgt
 4801 attccgcccc cgcccccgac gccagcttcc cctcagcct catgtccacc gacaacaca
 4861 ccaactctt cgagtgcgc agcaccctct acgccccggc cgtcgtcaac gatctgtgag
 4921 ggtctgggag ccttgttagc gatgtctaac cgaataaag ggtcgaaac ggactgttgg
 4981 gtctccggtg tgattattac gcaggggagg ggggtggcgg ctggggaaag ggaagggaacg
 5041 cccgaaccca gagaaaagga ccaaaagga aacgcgtcca accgataaat caagcgccga
 5101 ccagaacccc gagatgcata ataacaacg attttattac tcttattatt aacaggtcgg
 5161 gcatcgggag gggatggggg cgcgcgttcc ctccgttcgg gctactcgtc ccagaattta
 5221 gccaggacgt ccttgtaaaa cgcgggcggg ggcgcgtggg cccacacctg cgccagaaac
 5281 cggtcggcga tgtccggggc ggtgatatga cgaatcacga tggagcgcgc taaatcttcg
 5341 tcgcggagggt cctgatatagat gggcagtcctt tttagaagag tccagggtcc ccgctccttg
 5401 gggetgataa gcgatatgac gtacttgacg tatctgtgct ccaccagctc ggcgatggtc
 5461 atcggatcgg gcagccagtc caggccctcc ggggcgtcgt ggatgacgtg gcggcgacgt

FIG. 12D

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5521 ccggcgacat agccgcgggtg ttccgcgacc cgctgcgcgt tggggacctg caccgagctcg
5581 ggccggggtga gtatctccga ggaggacgac cgggcgcctt cgcgcggccc accggcgacg
5641 tccgggggct ggaggggggg gtcttcttcg tagtcgtcct cgcgcgcgat ctgttgggcc
5701 agaatttcgg tccacgagat gcgcgtctcg aggcgcgacc gggccgcggt cagcgtaggc
5761 atgctctcca gggagcgca gttggcgcgc tccgcgcggg ccgcccggcg ggcctgggat
5821 cggctcgggg cggtcagtg aactcgcgc agcagtcct cgcgcgacgc gtagggtgta
5881 ttggggtgca ggtctgtgtg gcagcggacg aacagcgcca ggaactgcgg gtaactcatc
5941 ttgaagtacc ctgcag

FIG. 12E

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1 aaaccactgt tctttacaact ttatgctcta gtttttggta atagtgtctt ggaacacttt
 61 taccctaacc gaattatgg ctttggattt tttagacacc gactgtccac tggggattgt
 121 ttecgatatt atatecaacg tgaataccat caaagagtat ggatatcca gcgaattatc
 181 aacaaacgctg gcacctcgcc cgtctcgaga acagggttta gagtatatca ccagagtcgt
 241 ggataaacac aagecgctgt gcagagtcga cgaacgcctt tacattgcgt gcggggagct
 301 tgtacaccta cgaattaaag caegcaaac agacctgaaa tattggctaa aatcgctctga
 361 gattgatctt agcgatgtcg tggaaacaggc catattggaa cacattgact ttgttcagaa
 421 aacctcaac tcgtttgaaa catcggaata ccgagatttg tgttcattag gcctgcaatc
 481 tgcgctaag tatgaagaaa tgtatttagc caaatgca ggcggacgct tagagtccat
 541 ggggcaattt ttcttagac ttgcaactac tgetacgcac tatactatgg acaaccagc
 601 aatggctcgc gtgttggta gcggtgaggt tggctggaca tatatttca gacctttt
 661 tactgcgcta gccggacagg ttgtcattcc gcccacgcca attatgctgt ttggtgggag
 721 agactgtggg tctatggcca gctgttattt gctaaacccc agggtaacag atatgaactc
 781 tgaattccg gctcttatgg aagaggttgg acccattttg tgcaaccgag gaggaattgg
 841 actgtcttta cagaggttta aacctccacc cacagaaggt tgttcacggg gtgtcatggc
 901 tctcctaag ctactagact ctatgacct ggccattaac agcgacggtg aaagaccaac
 961 aggagtgtgt gtttatctcg aacctggca cgcagacatc cgcgccattt taatatgcg
 1021 cggaatgctg gccagagacg aaactgtgcg ctgcgacaac atctttgctt gtatgtggac
 1081 ccagacctg ttttttgacc gctatcaacg gtacgtcgat ggagaaagcg gcataatgtg
 1141 gactctgttt gatgatactg catcgacct ctgccatatg tacggaaatg atttcacacg
 1201 ggaatatgag cgcctggagc ggtgtggatt tgggatagac gctattccca tacaggacat
 1261 ggcctttatc atagttagaa gtgctgtaat gacaggaagc ccatttttga tgtttaaga
 1321 cgcgtgcaac aggcactacc actttgacat gcggcagaga ggtgcgataa tggggtctaa

FIG. 13A

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1381 tctatgcaca gaaattatcc agcatgccga cgaaccccaa aacgggggtgt gtaatctagc
 1441 cagcatcaac ctccc aaat gtctagccct tccacctcca aatattgcag gtgtgccata
 1501 ttttgacttc gccgctctgg gccgcctgc cgcactgcc acaatttttg tcaatgcgat
 1561 gatgtgtgcc agcacatate caactgttaa atcccagaaa ggcgltgaag aaaaccggtc
 1621 gctgggactt ggaattcagg ggctacatac caegttttg atgctggacc tggatatggc
 1681 atctccagag gcgcaccaac taacaagca aatagcaga aggctgttat tgaactctat
 1741 gaaggccagc gcaacgtctt gcaagctggg tatgcaaccc tttaaaggtt ttgaagacag
 1801 caagtacagt cggggggaac taccctttga tgcctacca aatgtaaac taacaacccg
 1861 caacgcctgg cgtagacttc gcaatgacat aaacaatac ggcttgtaca attctcagtt
 1921 tgtagectat atgccaacag tatcttcgtc acaggttacc gagagcagcg aggggttttc
 1981 tcctgtttac acaaacctgt tttagcaagt tactgtacc ggggaagtac tcaggcccaa
 2041 tgtactgcta atgcgcacca tcagaagtat ttttccacag gaatgcgcgc gttacaagc
 2101 gctatcttacg ctagaagctg cgaatggtc agttgtggga gcgtttggcg atttgccagt
 2161 tggteacccc ctcaagtaagt ttaaacacagc atttgagtac gaccagacta tgctaattaa
 2221 catgtgtgct gacagggtcg cgtttgtgga ccagagccaa tccatgtctt tgtttataac
 2281 tgagcctgct gacgggaaac tccccgcctc cagaattatg aatcttttgg tccacgcata
 2341 taacgcgga cttaaaacag gcatgtacta ctgcaaaatc aagaaggcaa caacaacgg
 2401 agtctttgtt ggcggagacc tagtctgca cagctgcagc ttgtagggca gcctcgccat
 2461 ttgtcccagg gcgggaaat aattatggcc ctgaaact ctaaaaaac agattttgct
 2521 gacgagttat tgataaatgc gtatttctat acgccggaat gtcccgatat tgaacaccta
 2581 cgcttggtga gcgttgccaa ccgctggctg gatacggacc ttccaatttc tgatgacctc
 2641 aaggacgttg ctaaactcgc gccagccgag cgagagtttt accggttttt gtttgccttt

FIG. 13B

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2701 ttatctgctg ctgacgactt ggtaaatlta aacctgggag atttatcegc actatttact
2761 caaaggaca ttcttcacta ctacattgag caagagtcta ttgaagtaac gcactccaga
2821 gtatatagcg ctatacagct tatgttgttt ggaaacgacg caacagcgcg cgctagggtat
2881 gtcgcactcg ttgtcaaga cgtggccata gacctaaagg tatcttggtt gcaagcaaaag
2941 gtgcgagaat gcaaatctgt ggcggaaaaag tatattttga tgatattaat agaggcggtt
3001 ttcttcgcgt cgtcctttcc gtccatcgca tatcttgcga cccacaatct ctttgttgta
3061 acctgtcaaa gtaatgatlt aattagccgc gacgaagcaa ttcaacccaa cgcctcgtgc
3121 tgtatctaca caactacct tgggcgtttt gaaaagccag ctccaacgag gatttatgcg
3181 ctgttttctg aggccgtaaa catcgagtgt gaatttttgc ttcccatgc ccccaaaagc
3241 agccacctgt tggacattga agccatcata tgcctacgtac gctatagcgc ggacaggctt
3301 ttgggggaaa ttggactatc tccgctgttt aatgctccca aacccccacc aagcttcccc
3361 ctacgtttca tgactgtgga aaacatatcc aacttttttg aaaggcggaag caccgcatac
3421 tcgggaactc ttataaacga tctgtaatgt aaaaataaaa actaattttg attcaattat
3481 ttgtcttgtt tgcgtgttgg atgtacgcga tttaaaaaaa tactgagaaa agatactccc
3541 gattttaactt tatttaagac cattgtcttc ggtgtccaca gtcatcccg tagttaacca
3601 acacagtgtt gtaatcagtg ggggtgggaa tgtggttcca aaacatatata gcaagctctc
3661 tgacaatttc gtgttcgg

FIG. 13C

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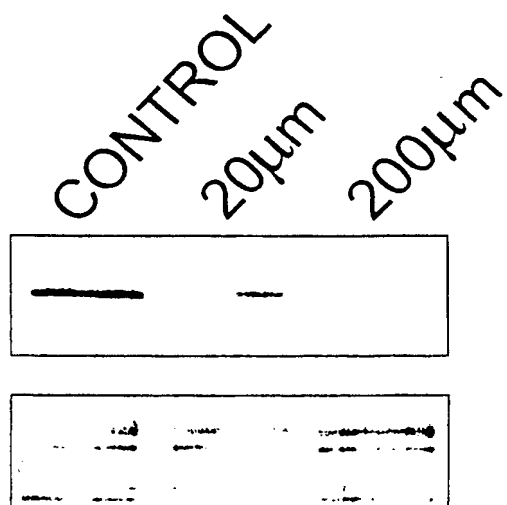
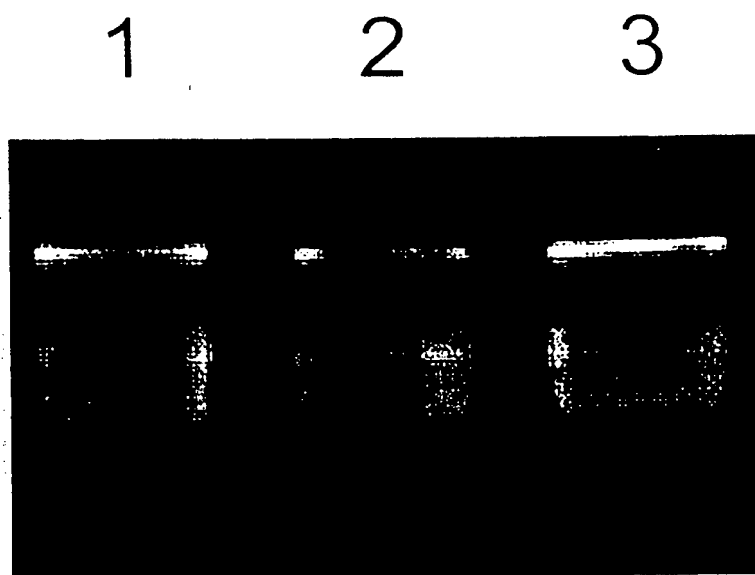


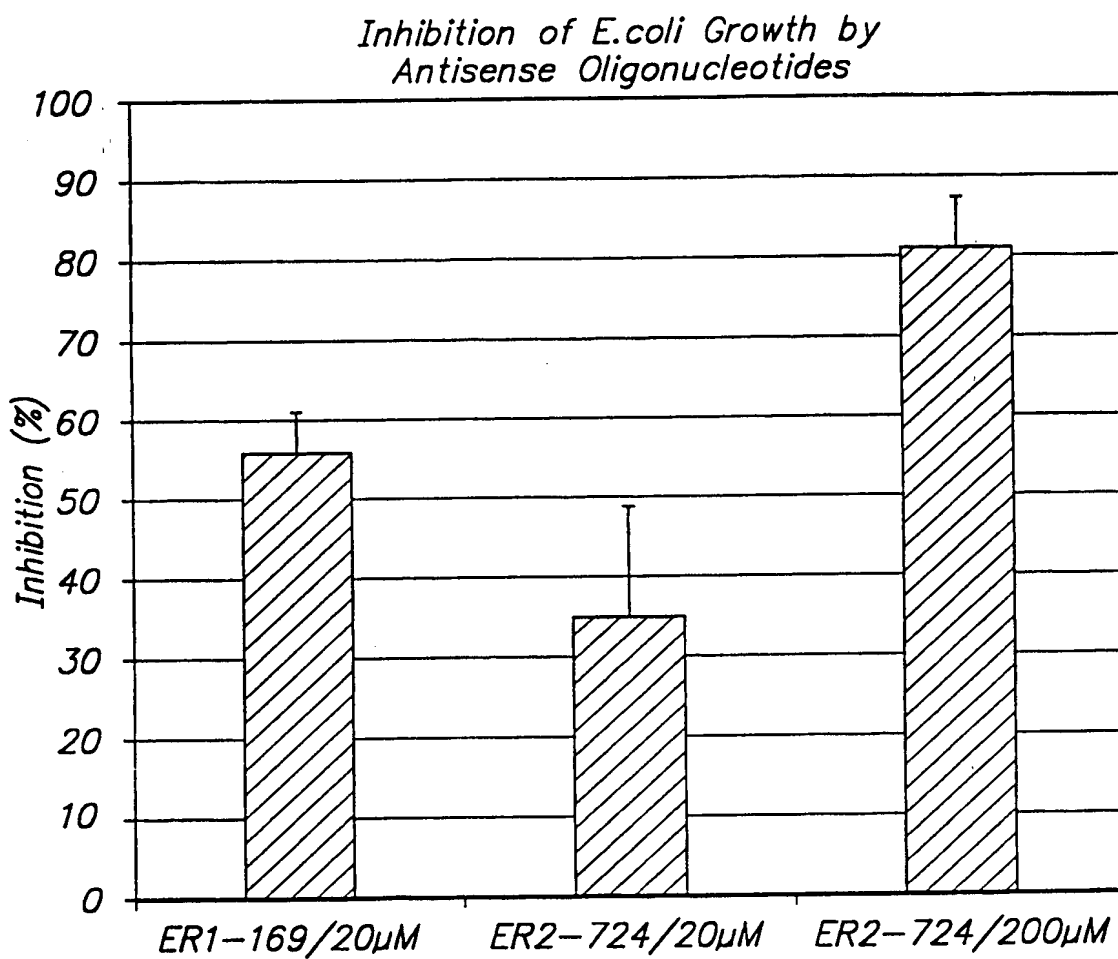
FIG. 14



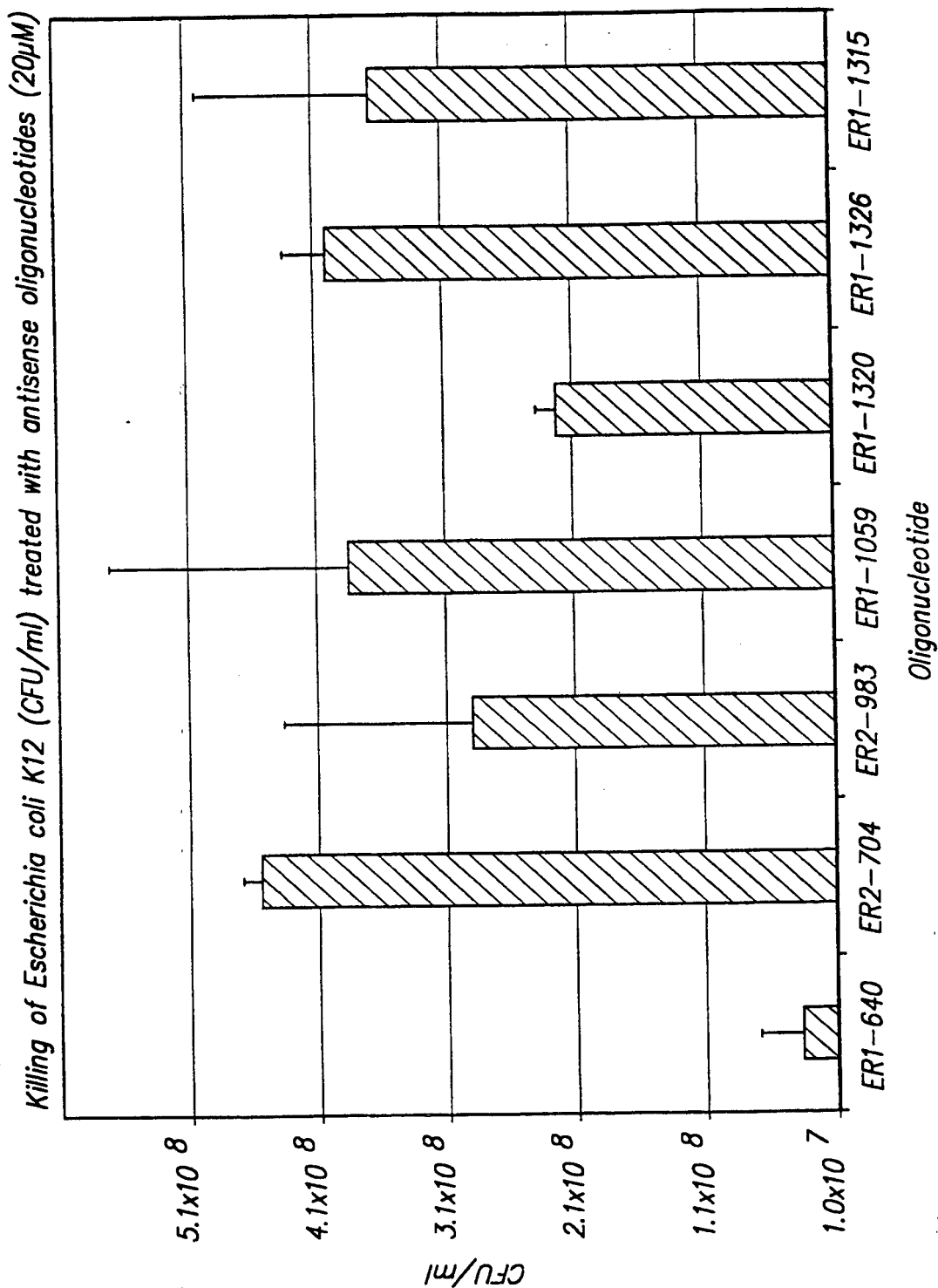
SecA

FIG. 17

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**FIG. 15**

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**FIG. 16**

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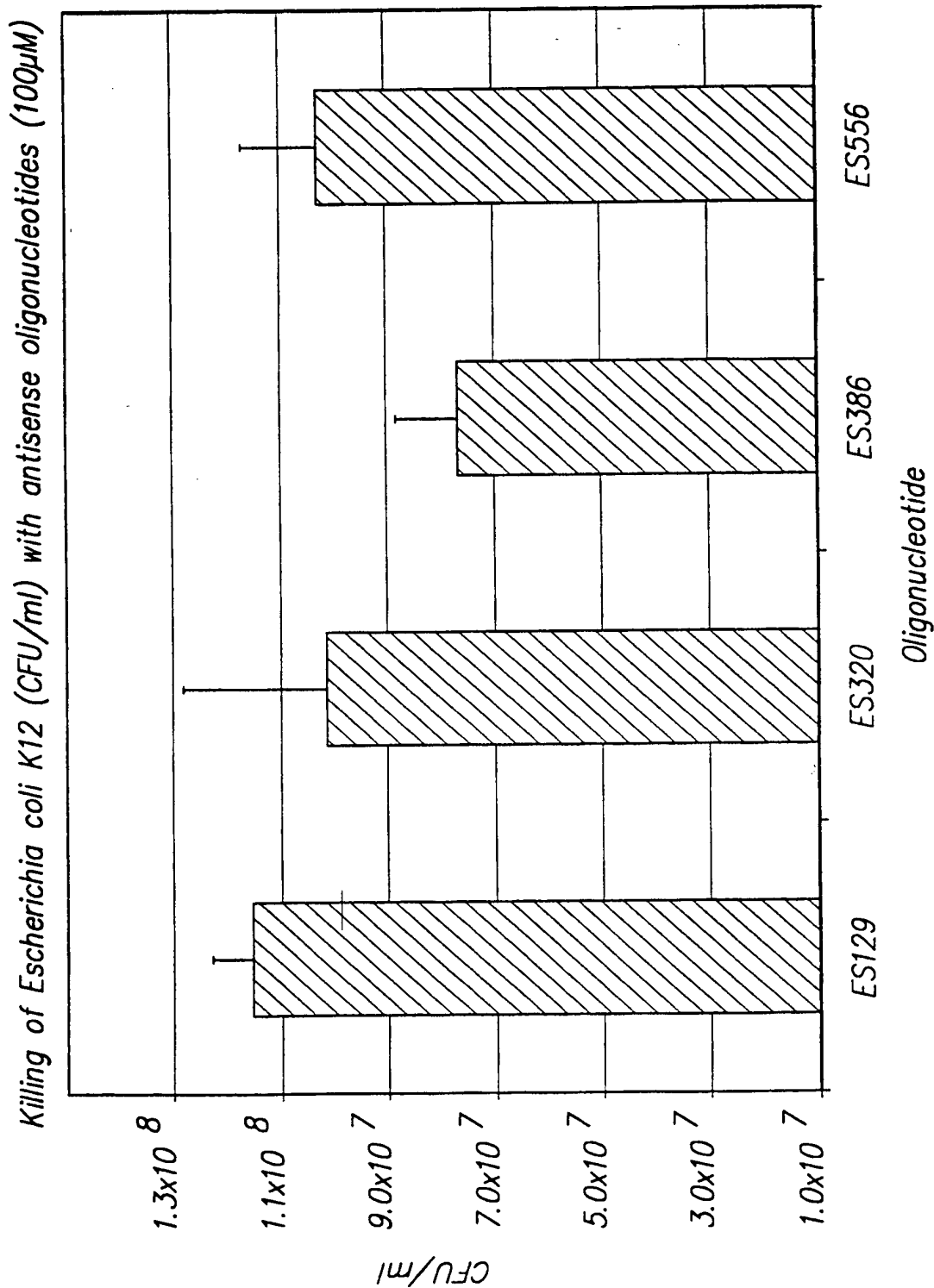


FIG. 18A

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Killing of Escherichia coli K12 (CFU/ml) with antisense oligonucleotides (20µM)

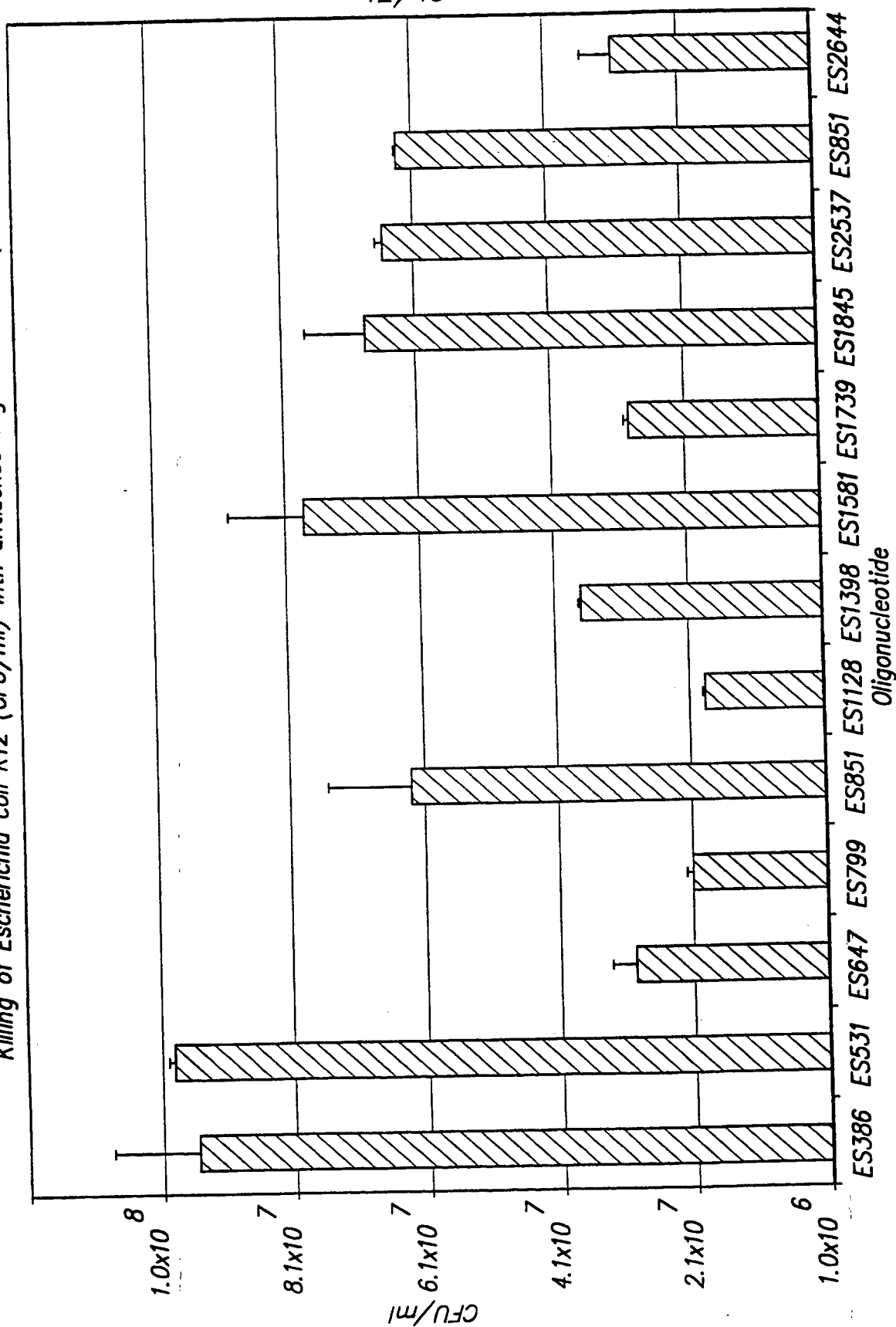


FIG. 18B

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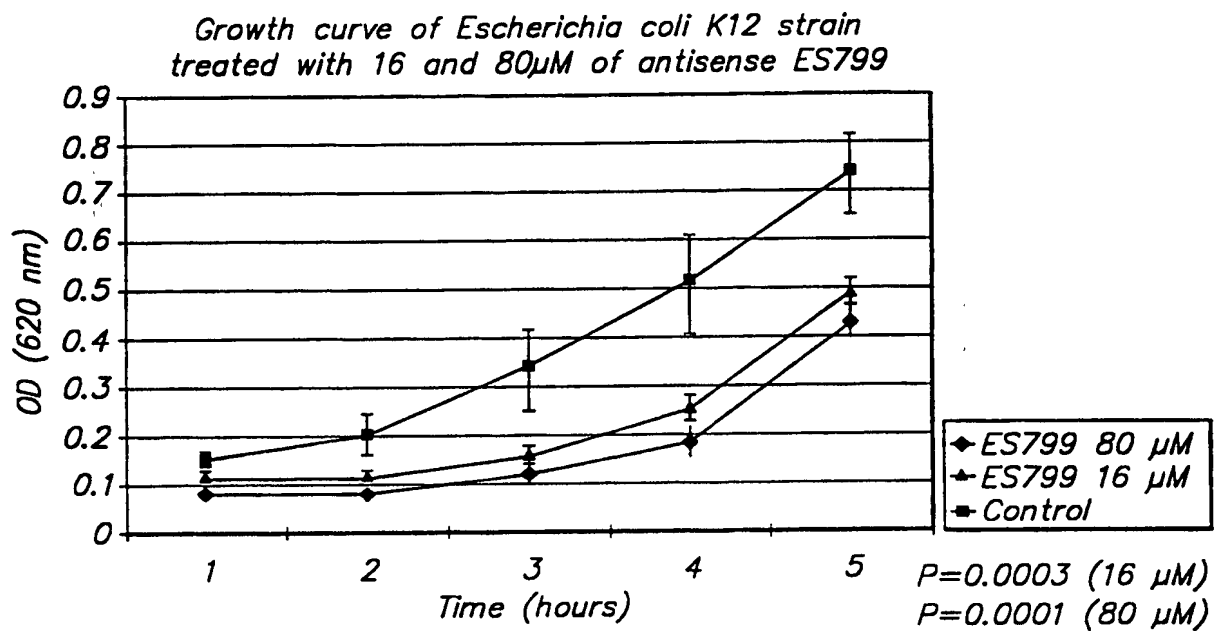
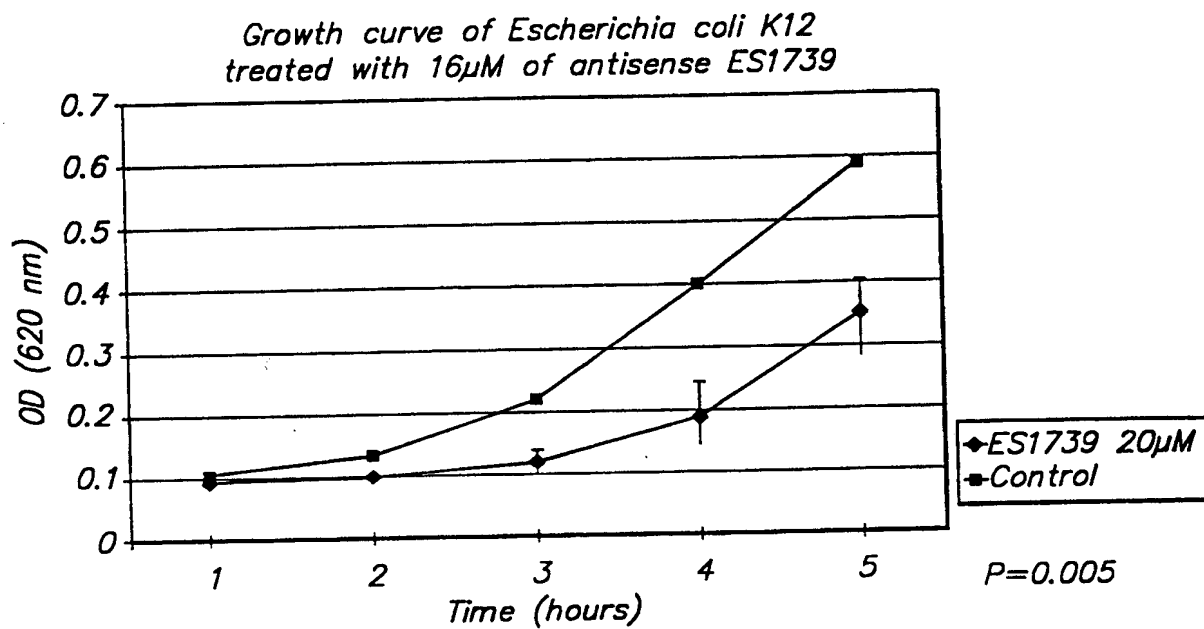
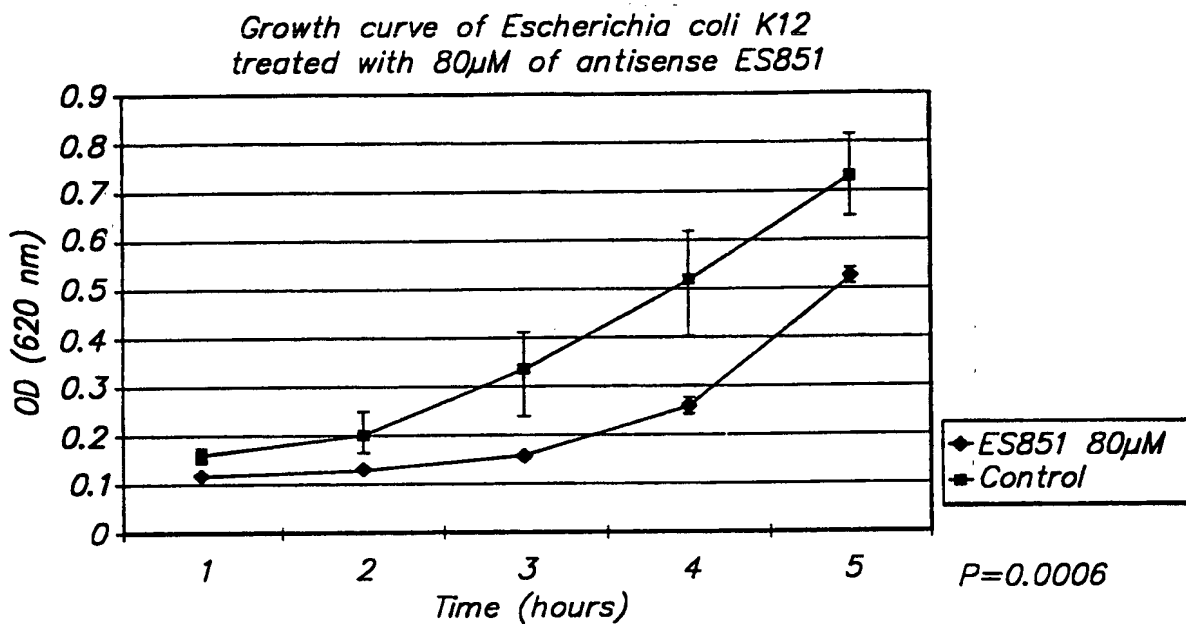


FIG. 19A

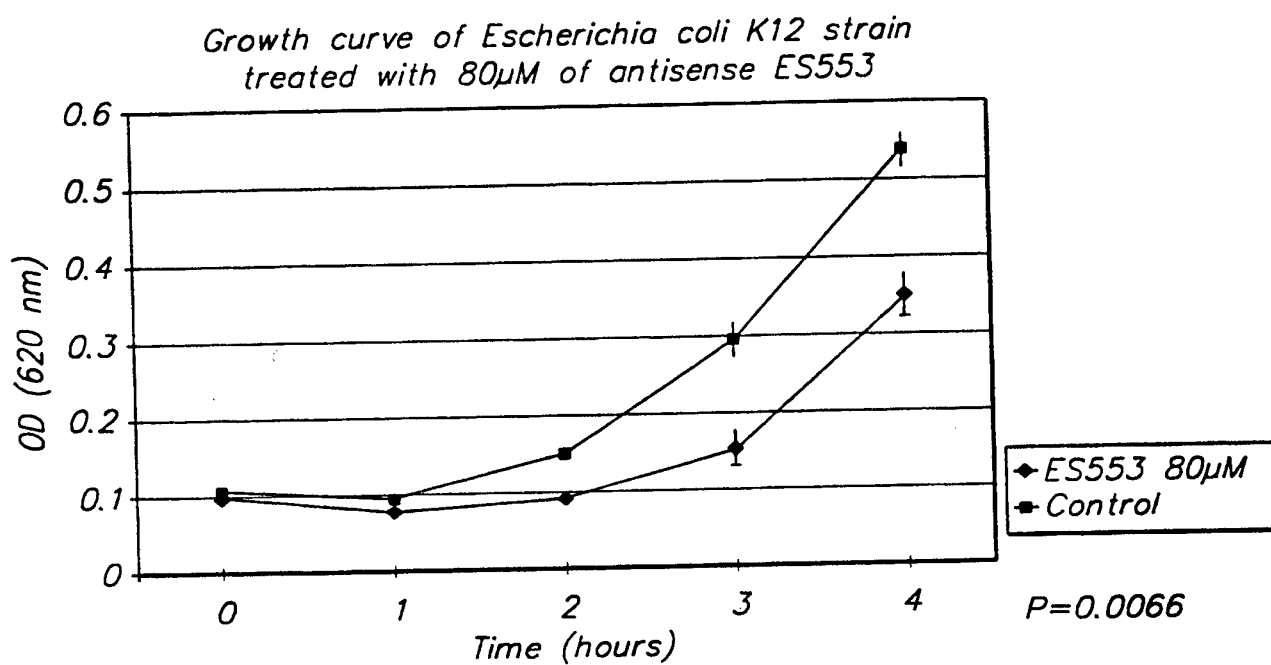
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**FIG. 19B**

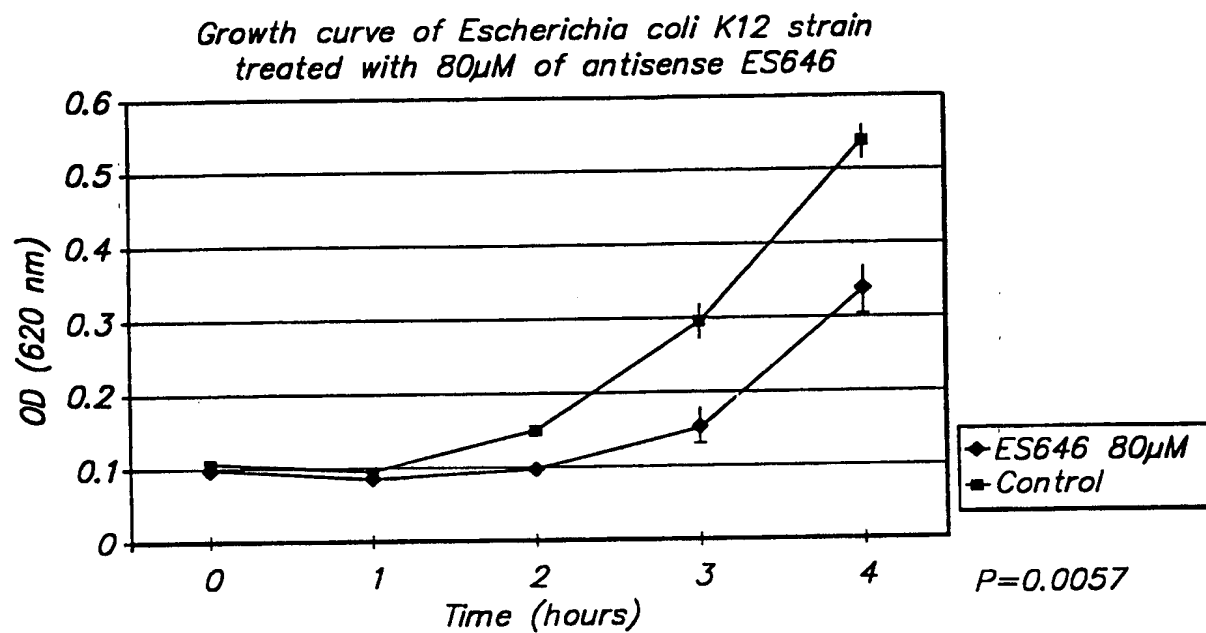
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**FIG. 19C**

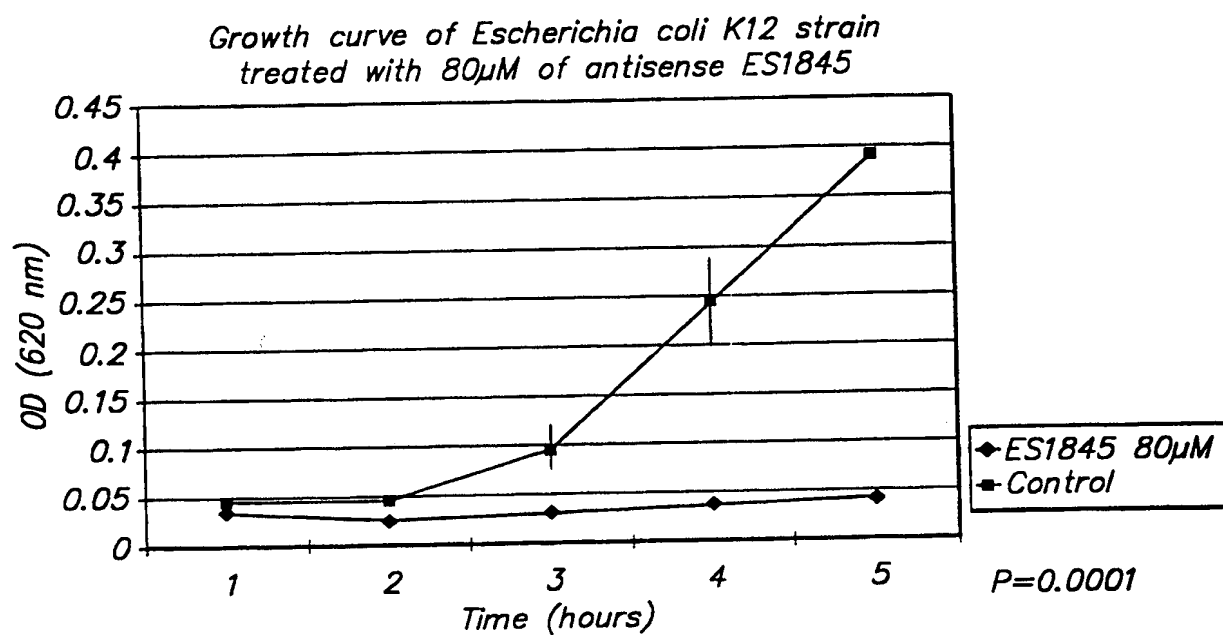
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**FIG. 19D**

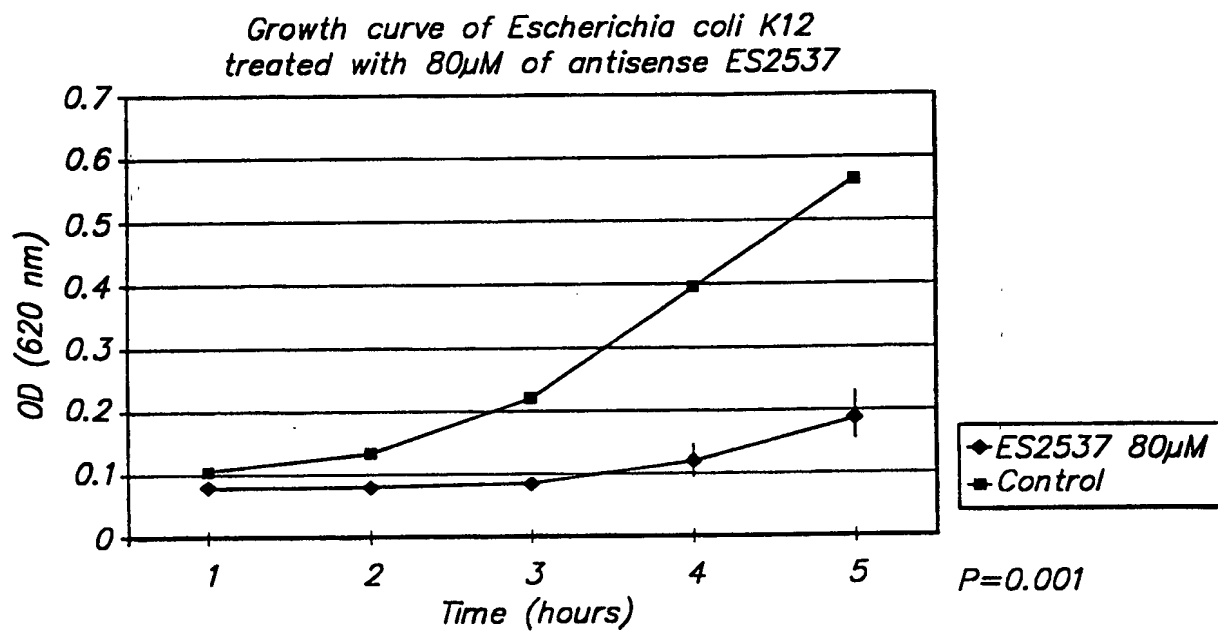
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**FIG. 19E**

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**FIG. 19F**

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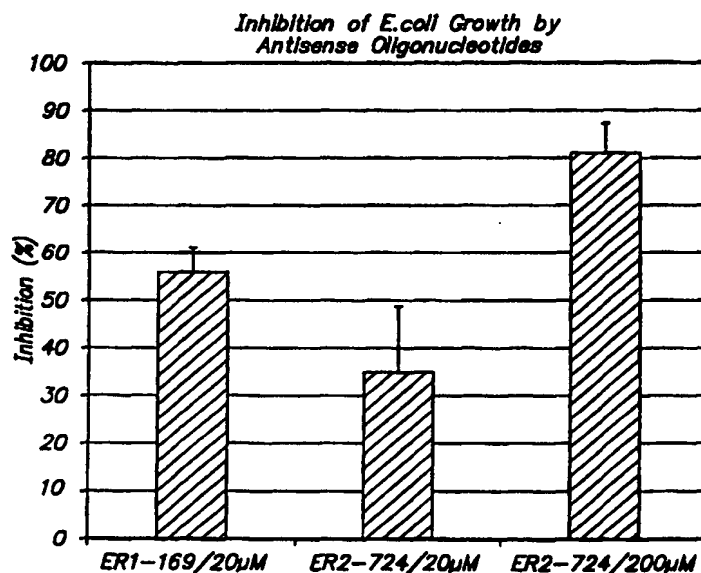
**FIG. 19G**



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(22) International Filing Date: 10 July 1998 (10.07.98)			
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(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 60/052,160 (CON) Filed on 10 July 1997 (10.07.97)		Published With international search report.	
(71) Applicant (for all designated States except US): GENESENSE TECHNOLOGIES, INC. [CA/CA]; Sunnybrook HSC, Room S-115, 2075 Bayview Avenue, Toronto, Ontario M4N 3M5 (CA).		(88) Date of publication of the international search report: 1 April 1999 (01.04.99)	
(72) Inventors; and (75) Inventors/Applicants (for US only): WRIGHT, Jim, A. [CA/CA]; Apartment 902, 5418 Yonge Street, Toronto, Ontario M4N 6X4 (CA). YOUNG, Aiping, H. [CA/CA]; Apartment 508-88 Grandview Road, Toronto, Ontario M2N 6V4 (CA). DUGOURD, Dominique [CA/CA]; 2053 A Mt. Pleasant Road, Toronto, Ontario M4P 2M5 (CA).			

(54) Title: ANTISENSE OLIGONUCLEOTIDE SEQUENCES AS INHIBITORS OF MICROORGANISMS



(57) Abstract

The invention relates to antisense oligonucleotides which modulate the expression of the ribonucleotide reductase or the secA genes in microorganisms. This invention is also related to methods of using such oligonucleotides in inhibiting the growth of microorganisms. These antisense oligonucleotides are particularly useful in treating pathological conditions in mammals which are mediated by the growth of microorganisms.

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 98/00666

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/11 C12N15/31

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO 98 05769 A (GENESENSE TECHNOLOGIES, INC.) 12 February 1998 *see the whole patent* ---	1-17
Y	F.R. BLATTNER ET AL.: "The complete genome sequence of E. coli K-12" SCIENCE, vol. 277, 1997, pages 1453-1462, XP002089422 *see the whole article* ---	1-17
Y	WO 96 26276 A (THE GOVERNEMENT OF THE UNITED STATES OF AMERICA ET AL.) 29 August 1996 *SEE THE WHOLE PATENT* --- -/--	1-17

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Date of the actual completion of the international search

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International Application No

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Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	W.J. PHILIPP ET AL.: "An integrated map of the genome of the tubercle bacillus, M. tuberculosis H37 Rv, and comparison with M. leprae" PROCEEDINGS OF NATIONAL ACADEMY OF SCIENCES USA, vol. 93, 1996, pages 3132-3137, XP002089427 *see the whole article* ---	1-17
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INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 98/00666

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Y	B. BEALL ET AL.: "Sequence analysis, transcriptional organization and insertional mutagenesis of the envA gene of E. coli" JOURNAL OF BACTERIOLOGY, vol. 169, 1987, pages 5408-5415, XP000350648 *see the whole article*	1-17
Y	A. JORDAN ET AL.: "Cloning and sequencing of the genes from S. typhimurium encoding a new bacterial ribonucleotide reductase" JOURNAL OF BACTERIOLOGY, vol. 176, no. 11, 1994, pages 3420-3427, XP002089431 *see the whole article*	1-17
Y	A. JORDAN ET AL.: "The ribonucleotide reductase system of L. lactis" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 15, 1996, pages 8779-8785, XP002089432 *see the whole article*	1-17
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International Application No

PCT/CA 98/00666

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Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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